A B S T R A C T

*Background:*Simian virus 40 (SV40) is a small DNA tumor virus found associated with specific human cancers, such as brain and bone tumors, malignant pleural mesothelioma, and non-Hodgkin lymphoma. SV40 sequences were also detected, although at lower prevalence, in blood specimens from healthy donors. However, some studies failed to reveal the presence of SV40 in human samples, and its association with specific neoplasms. These conflicting results indicate the need of a specific standardized test, to be share within the scientific community, to study the SV40 infection in humans and its association with cancers.

Methods: In this investigation we report on the presence in human sera of antibodies reacting against specific SV40 peptides revealed by an indirect ELISA. test. The immunologic assay was set up employing synthetic peptides corresponding to SV40 epitopes of the viral capsid proteins and the large T antigen (Tag), the SV40 oncoprotein. Sera from healthy donors and oncologic patients were tested with indirect ELISA assays. Overlapping results were obtained with this immunologic assay employing four specific peptides from early and late SV40 regions.

Results: Data from immunologic assays indicate that in healthy individuals serum antibodies against SV40 are detectable since the early childhood. In healthy donors the seroprevalence increases with the age reaching the peak of 25% in subjects aged 1-10 years, it declines in subjects aged 11-17 with a prevalence of 15%, then it slightly increases to 20%, remaining stable till the age of 50 years old. In the cohort of individuals aged 51-65 year old the seroprevalence tends to decline with a prevalence 17%, while in elderly subjects aged 66-90 years the prevalence is the 23%. This result indicates that the SV40 circulation in humans is of low level compared to the prevalence of the closely related human polyomaviruses BK (80-90%) and JC (60-70%). In our investigation, serum samples from oncologic patients affected by bone and brain tumors, non-Hodgkin lymphoma, and malignant pleural mesothelioma, had a high prevalence of anti-SV40 antibodies.

Conclusions: In our study, the prevalence of serum samples reacting with SV40 peptides was higher in sera from oncologic patients, compared to controls. Indeed, SV40-positive sera were detected at higher prevalence in patients affected by osteosarcoma (57%), non-Hodgkin lymphoma (51%), glioblastoma (34%) and mesotheliomas (32%) than in normal subjects (18%). No statistically significant difference in prevalence was revealed in sera from patients affected by breast cancer (21%) and undifferentiated nasopharyngeal carcinoma (25%), a neoplasm associated with the EBV infection, compared to samples from healthy donors. Our data further indicate the association of SV40 with specific human malignancies.

Chapter 1

Introduction

1.1 Simian virus 40

Simian virus 40 (SV40) was assigned to the family of Papovaviridae, an acronym proposed by Melnick and obtained by fusing the names of the three representative viruses Papilloma, Polyoma, and Vacuolating agent. More recently, SV40 has been considered a Polyomavirus, together with the human BK (BKV) and JC (JCV) Polyomaviruses. The virion is about 45 nm, an icosahedral particle, with a density of 1.34-1.35 g/cm^3. The viral genome is a circular, double-stranded DNA molecule.[1-3] The DNA is complexed with histones, forming a nucleosomal structure similar to cellular chromatin, referred to as a minichromosome.[4] Genome is small (5.2 kb) and contains a limited coding capacity (Figure 1.1). It comprises three parts a non-translated regulatory region of about 400 base pairs (bp) in size that contains the origin of replication (*ori*), the promoters and enhancers that control replication; the early region that encodes the

replication proteins (Tag, tag and 17 KT protein) is expressed soon after the virus enters a cell; and the late region that encodes the capsid proteins (VP1, 2 and VP3) and a maturation protein (agnoprotein), and is expressed efficiently only after viral DNA replication has begun. The transcriptional promoters and enhancers are located very close to the functional *ori* sequence. The early promoter contains three Tag-binding sites, some of which overlap the TATA box element that directs the initiation of transcription. Immediately adjacent is a GC-rich cluster (21-bp repeats) that binds the transcription factor SP1. Further upstream is the enhancer region (72-bp element), which contains several transcription-factor-binding sites and acts to increase transcription initiation. Viral transcription is mediated by cellular RNA polymerase II. Only small amounts of early transcripts are produced (a few hundred molecules per cell), whereas late transcripts are much more abundant (several hundred thousand copies per cell). Early and late transcription proceeds bidirectionally from near the *ori*, with the early and late transcripts being produced from opposite strands of the viral DNA. Alternative splicing of pre-messenger RNAs (pre-mRNAs) produces functional mRNAs. TAg is not a transcription factor *per se*, but it can autoregulate the early promoter as the replication cycle proceeds. When Tag reaches a high enough concentration in the cell, it binds to viral DNA which might block the assembly of functional transcriptional complexes, repressing early transcription. Tag indirectly contributes to the activation of SV40 late transcription in ways that are not clear, perhaps by stabilizing interactions among transcription factors. Viral DNA replication requires a functional SV40 *ori*, Tag protein with intact DNA-binding and helicase activities, and several cellular proteins involved in DNA synthesis. Tag binds to specific sites in the SV40 *ori*, catalyses local unwinding of the viral DNA, and recruits cellular DNA replication proteins to the complex, including topoisomerase I, replication protein A (RPA) and DNA polymerases. The nature of cellular DNA polymerase primase from different species limits the host range of polyomaviruses. Both monkey and human proteins can replicate SV40 DNA. Topoisomerase II separates the newly joined replicated circular DNA daughter molecules.[5]

Ori encompasses the target for Tag helicase activity and the origin of bidirectional DNA replication.[6,7] All other proteins involved in DNA replication, DNA transcription and chromatin assembly are provided by the host cell. *Ori* consists of *ori*-core and two

ori-auxiliary sequences, aux 1 and aux 2. *Ori*-core is the minimal cis-acting sequence required for replication under all conditions in vivo or in vitro. Aux 1 and aux 2 are non-interchangeable sequences that flak *ori*-core and facilitate its activity in vivo and in vitro.[8-10] Aux 2 contains several transcription factor binding sites as well as weak Tag binding sites and is required both for early gene promoter activity and for maximum *ori* activity. Aux 1 contains a strong binding site for Tag referred to as Tag binding site I. Mutations in Tag binding site I then diminish Tag binding also diminish activity of *ori*-core, although they stimulate activity of the early gene promoter, suggesting that Tag binding to aux 1 simultaneously represses transcription and facilitates replication.[11-13]

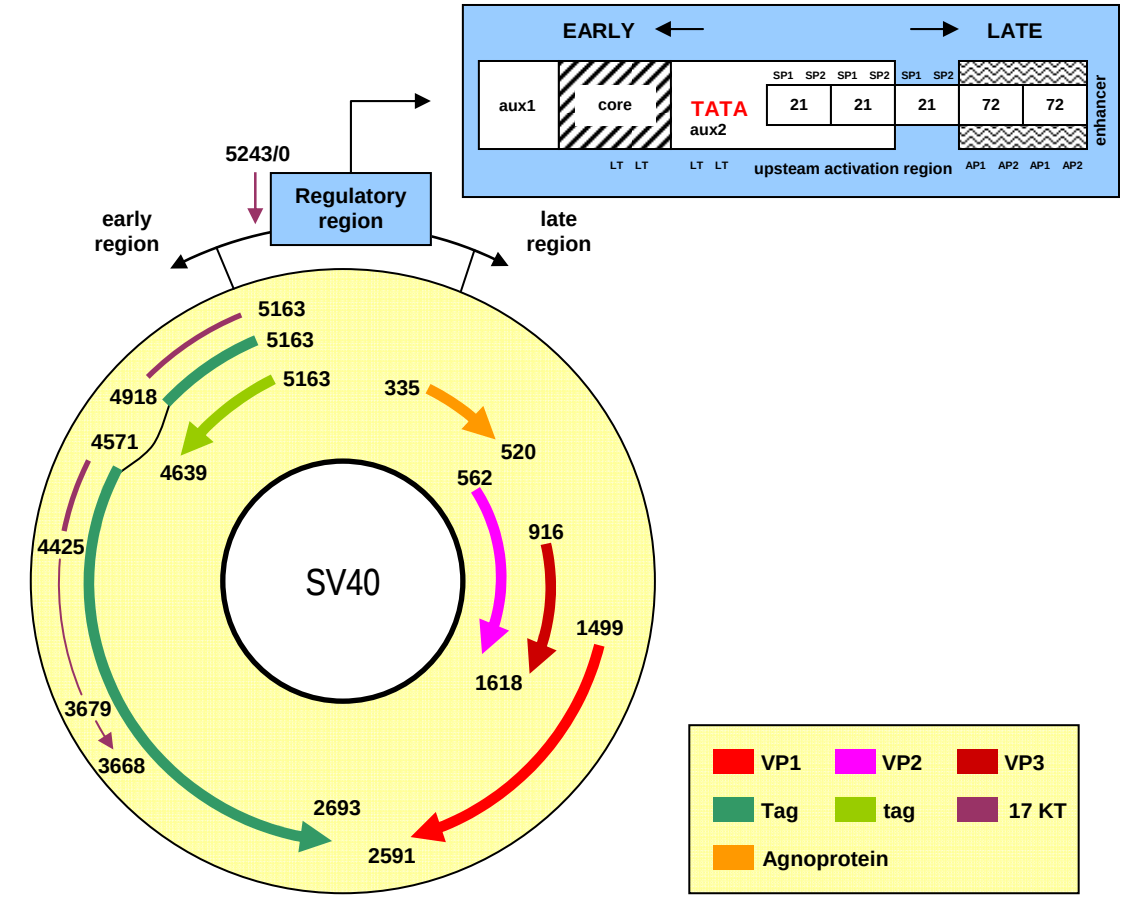


Figure 1.1 Genetic map of SV40. The thick circle represents the circular SV40 DNA genome. Nucleotide numbers begin and end at the origin (*ori*) of viral DNA replication (0/5243). The open reading frames that encode viral proteins are indicated. The early Tag proteins are shown on the right, and the late structural (VP) proteins are shown on the left. The beginning and end of each open reading frame are indicated by nucleotide numbers.

1.2 Capsid proteins of Simian Virus 40

The SV40 capsid, whose structure is known at atomic resolution, is built from 72 pentamers of the major capsid protein VP1 and 72 total copies of the minor capsid proteins VP2 and VP3. One of either VP2 or VP3 resides in the central cavity of each VP1 pentamer. (VP2 and VP3 are identical, except for the additional amino-terminal segment of VP2).[14-16] Enclosed in this protein shell is the viral minichromosome, a condensed complex of the 5.2-kbp circular double-stranded viral DNA and the four host-derived core histones, H2A, H2B, H3, and H4 (Figure 1.2).[17]

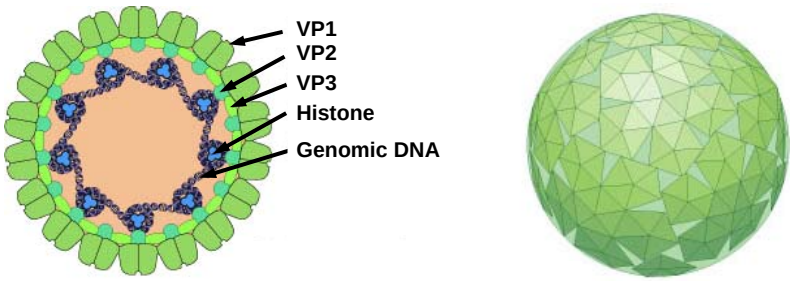


Figure 1.2 Representation of structural proteins and minichromosome of SV40.

The assembly of VP1 pentamers into icosahedral shells is an intrinsic property of VP1, since VP1 alone of SV40 and several other polyomaviruses is sufficient for the formation of capsid-like structures in vitro, in Escherichia coli, in Saccharomyces cerevisiae, in insect cells, and in mammalian cells.[18]

Furthermore, the capsid proteins of polyomaviruses have been implicated, by numerous studies using different experimental systems, in other important functions of viral infection. During early stages of infection, the VP1 capsid mediates interaction with cell surface receptors, leading to cell entry; the N-terminal domain and myristoyl modification of VP2 allow proper interaction of internalized particles with host membranes or structures; the C-terminal domain of VP1 may mediate binding to the membrane of the endoplasmic reticulum (ER); the VP1 calcium-binding sites may control capsid conformational change in the cytoplasm, leading to the exposure of minor capsid proteins; the VP3 nuclear localization signal (NLS) mediates the nuclear entry of SV40 virion DNA; and the interaction of VP1 with poly(ADP-ribose) polymerase (PARP) may regulate the expression of viral early genes.[19-21] During late

stages of viral infection, minor capsid proteins may influence the proper folding and phosphorylation of VP1; the SV40 VP1 and VP3 DNA-binding domains are believed to be involved in viral genome packaging; VP2/3 have an inherent lytic property that may lead to host membrane permeabilization and facilitate virus exit, and this host necrosis may also be induced by stimulation of PARP activity by VP3.[22,23] Although VP2/3 are essential viral gene products and have been implicated in many of the above-mentioned infection processes, it has not been clearly established whether VP2/3 are required for some or all of these processes in the context of SV40 infection of host monkey kidney cells.[24] Based on a structural model, we previously identified residues of SV40 VP1 and VP3 that, when mutated, selectively disrupt the interaction of the two proteins.[25] The analysis of three VP3 mutants (F157E-I158E, P164E-G165R-G166E, and P164R-P165E-P166R) and three VP1 mutants (V243E, L245E, and V243E-L245E) by transfection of the mutant viral genomes into host monkey kidney cells showed that all of the mutants replicate viral DNA and produce capsid proteins normally. However, the mutants are much less infectious than the wild type.[26] A plausible interpretation of these data is that the capsids of these particles comprise VP1 but little or no VP2/3 owing to compromised VP1-VP3 interaction. Since VP2/3 mediate the nuclear entry of the SV40 genome via interaction between the VP3 NLS and importins, particles lacking VP2/3 are predicted to be defective in infecting new host cells. Here, we provide evidence for the formation of nucleocapsids by SV40 mutants in which VP2/3 is either absent or nearly so. These particles consist of VP1 and the encapsidated viral DNA, along with core histones. The degree of loss of VP2/3 in the particles correlates with the reduction in viability, so that particles with no detectable VP2/3 are essentially noninfectious. Mutant particles are able to enter new host cells but fail to associate with importins and fail to express large Tag.[27]

There is a new SV40 gene product called VP4 that is expressed ~24 h after the late structural proteins VP1, VP2, and VP3. VP4 is essential for the timely lytic release of the viral progeny that enables the efficient spreading of SV40 in culture. VP4 oligomerizes with VP3, and when expressed together, these two proteins possess a lethal lytic property. What determines that late in the replication process, following viral assembly, SV40 expresses VP4 to initiate the death of the host cell and the efficient release of its progeny (Figure 1.3).

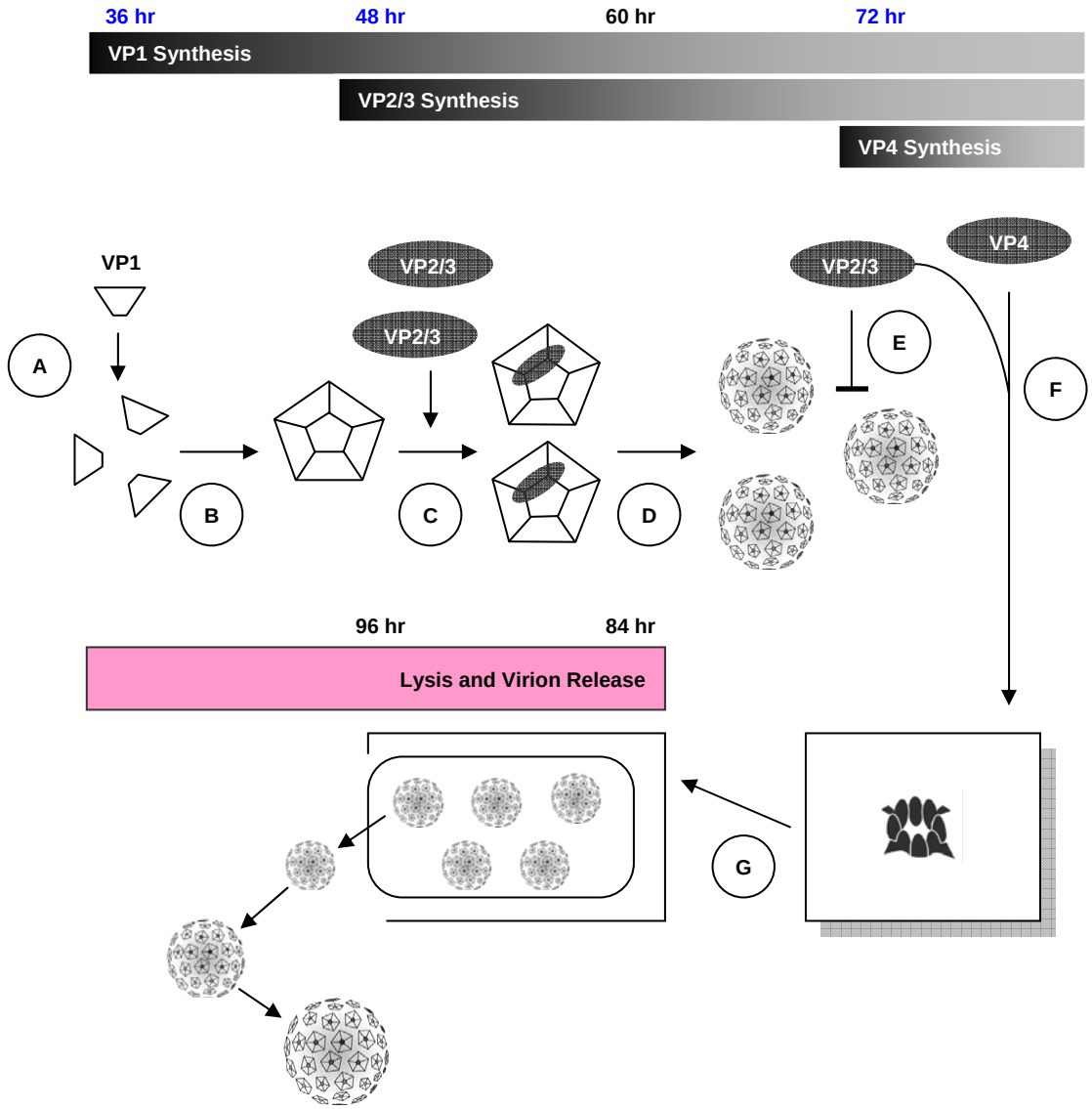


Figure 1.3 Proposed Model Displaying the Regulation of the SV40 Lytic Cycle. VP1 is synthesized first, forming pentamers that sequester newly synthesized VP2 and VP3 (steps A–C). Virion assembly (step D) and the stoichiometry of five VP1 per VP2/3 prevent VP2 and VP3 particle incorporation (step E). VP4 is synthesized, it oligomerizes with VP3, and possibly VP2, and targets to the host cell membrane to form a pore (step F), which then initiates the lytic death of the host cell and the release of the progeny (step G).

1.3 Non-structural proteins of SV40

Simian virus 40 picks the locks on pivotal check-points in the cell cycle control with the help of two proteins: Tag and tag, respectively. Upon infection, SV40 expresses these two proteins to achieve transformation of the host cell. The cell thus enters into an unchecked proliferation loop and turns into a factory for the replication of viral DNA and the production of viral particles.

1.3.1 Cellular transformation by SV40 Tag

Tag is a potent 708-amino acid oncoprotein capable of transforming a variety of cell lines and inducing tumors in various animal models. tag alone, on the other hand, cannot cause cellular transformation. It can, however, provide an important mitogenic signal that cooperates with the transforming activity of Tag. The transforming activity of Tag is attributed to its binding and manipulation of the function of certain key tumor suppressors and cell cycle regulatory proteins. The cellular target of Tag include the retinoblastoma family of proteins (namely, pRB and the closely related p107 and p130/pRB2), the tumor suppressor p53, and the transcriptional co-activators p300 and CBP.[28]

Transformation domains of Tag

The current model attributes Tag-mediated cellular transformation to three domains of Tag (Figure 1.4). (a) The J domain, residues 1-82, bears homology with the DNAJ family of molecular chaperones and binds to hsc70, a homologue of the heat shock protein hsp70. (b) The LxCxE motif, residues 103-107, binds to the retinoblastoma family of proteins pRB, p107 and p130. (c) A carboxy-terminal bipartite region, comprised of the amino acid 351-450 and 533-626, which binds to the tumor suppressor p53. This region has also been shown to be necessary for Tag association with CBP, p300 and the related p400.[29,30] These 82 residues of the J domain are identical between large Tag and small tag. The J domain cooperates with the LxCxE motif of large Tag to inactivate the functions of pRB family members and may also have additional transformation functions. Curiously, mutations disrupting the integrity of Tag's J domain without affecting the binding of pRB family members at the LxCxE motif, abolish certain effects of Tag on pRB proteins. Cellular transformation by Tag is mediated principally by the functional inactivation of cellular tumor suppressors pRB/p107/p130 and p53. Notably, the LxCxE motif of several viral oncoproteins, including SV40 Tag, adenovirus (Ad) E1A, and the human papilloma virus (HPV) E7, binds to pRB and pRB-related proteins p107 and p130.[31] Furthermore, SV40 Tag, Ad E1b (55kDa) and HPV E6 also bind to p53.[32] The transforming ability of these viral oncoproteins is abrogated by mutations that disrupt their binding with either pRB or p53. Besides the inactivation of important tumor suppressor proteins, SV40 Tag carries additional features that contribute to its transforming potential. Tag is equired for

SV40 viral replication. For this function, Tag provides ATPase and DNA helicase activities, as well as a specific DNA binding activity. These properties allow Tag to specifically bind to, and promote the unwinding of, the SV40 origin of replication, thereby helping in the replication of viral DNA. While none of these specific viral replication functions have been shown to contribute to Tag's transforming functions, Tag's DNA binding domain (residues 131-246) does appear to act as a transcriptional transactivator, possibly contributing to the transcriptional activation of certain cellular genes.[33] Another important interaction that may provide a link between Tag-mediated transcriptional activation and transformation, is the binding of Tag to p300/CBP.[34,35] p300 and CBP are members of a recently described family of transcriptional co-activators that cooperate with upstream cellular factors to activate transcription from a wide variety of promoters. Taking these additional findings into account, there appears to be ample evidence that other factors, besides the binding with p53 and retinoblastoma proteins, contribute to the full transformation potential of large Tag.

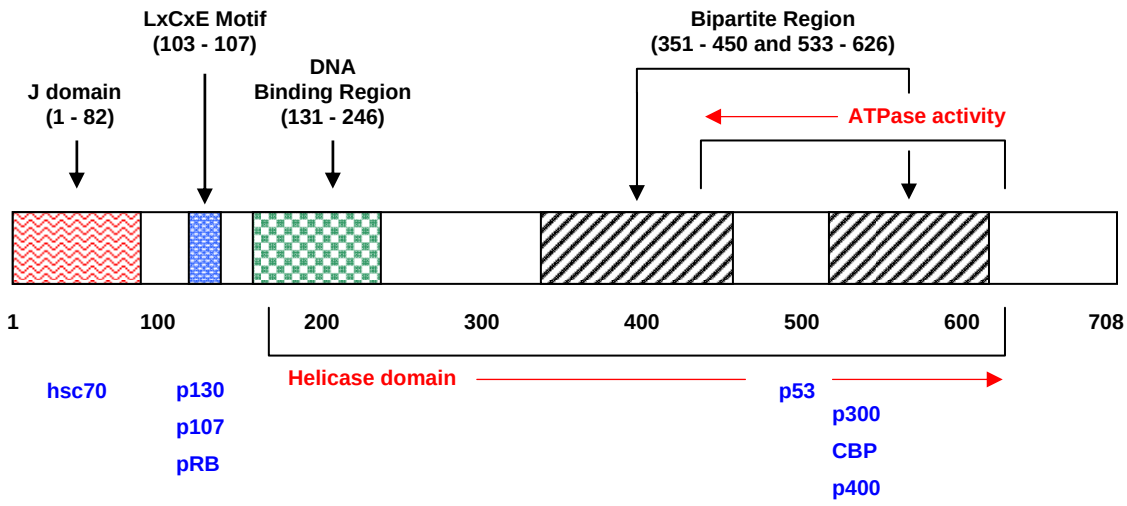


Figure 1.4 Transformation domains of SV40 Tag. Three major domains of Tag are known to be involved in its transformation function: the J domain, the LxCxE motif and the bipartite region, involved in Tag binding with, respectively, hsc70, Rb family members and p53. Two other regions that may contribute towards full transformation potential of Tag are its DNA binding and p300/CBP/p400 binding regions. The binding region for p300/CBP/p400 appears to be in the C-terminal region of Tag but there are indications that certain N-terminal residues may also play a role in this binding.

Interaction of Tag with host proteins

The following is a discussion of the biological functions, and the Tag-mediated deregulation thereof, associated with the targets of Tag, namely, pRB family members, p53, and p300 family members, known to be involved in Tag-mediated cellular transformation.

The retinoblastoma susceptibility gene, Rb-1, was the first tumor suppressor gene to be described. The pRB protein and its relatives p107 and p130 are critical for maintaining a tight control over cell cycle progression, thus controlling the vital processes of proliferation, differentiation and apoptosis.[36] Mutations in Rb-1 are frequently found in human tumors, establishing its role as a tumor suppressor. Further evidence corroborating the status of pRB as a growth suppressor comes from experiments performed with Saos-2 cells in which Rb-1 is mutated ($Rb^{-/-}$). The introduction of wild type pRB, p107 or p130 cDNA into this cell line results in growth arrest, emphasizing the role of pRB proteins in cell cycle control.[37,38] The regulation of cell cycle by pRB proteins is mediated, at last in part, through their binding to specific members of the transcription factor family E2F, designated E2F-1 through - 5. Each of these E2F species forms heterodimers with any of the two members of the DP family, DP-1 and DP-2.[39] The E2F-DP heterodimers bind to a sequence specific DNA site found in the promoters of several genes required for the entry of cell into S phase, such as cyclins A and E, dihydrofolate reductase, c-myc and c-myb.[40,41] When bound to specific promoters, the E2F-DP heterodimers serve both to active transcription as well as to recruit pRB family members where they serve to repress transcription. The pRB proteins undergo cell cycle-dependent phosphorylation. During the G1 to S phase transition, pRB is converted from its hypo- to hyper-phosphorylated state. Similarly, during the late G1 to S phase transition, p130 as well as p107 become hyperphosphorylated. The cyclin-dependent kinases, most notably cyclin D1/cdk4 and cyclin E/cdk2 contribute to the cell cycle-dependent phosphorylation of pRB, as well as p130 and p107. The cell cycle-dependent phosphorylation of the pRB family is thought to promote the dissociation of pRB proteins from E2F and relieve the pRB-inducend repression of E2F-mediated transcription. Due to their crucial roles in cell cycle control, manipulation of the function of pRB proteins allows viral oncoproteins to gain control of cell growth regulation and then exploit the host cell machinery for viral

growth and replication. SV40 large Tag preferentially binds to hypophosphorylated pRB, resulting in sequestration of the form of pRB capable of binding to E2F. This leads to an effect similar to the phosphorylation-mediated inactivation of pRB proteins (Figure 1.5).[42] Therefore, as Tag takes the active, hypophosphorylated pRB hostage, the check on E2F-initiated cell cycle progression is surrendered to the tyranny of Tag. The region of pRB proteins that binds to Tag is often referred to as the pocket domain (residues 373-772 in pRB). The tumor-derived, inactivating mutations of Rb-1 gene are commonly found in the pocket domain.[43] Not only do these mutations abolish the ability of pRB to induce growth arrest in Saos-2 cells, they also render it unable to bind to Tag.[44,45] This evidence supports the hypothesis that Tag targets an essential growth-suppressing function of pRB. The region of Tag that binds to the pRB family of proteins is the LxCxE motif, residues 103-107 (Figure 1.4). In addition, the N-terminal J domain of SV40 Tag has a role in pRB inactivation. Mutations that disrupt the integrity of the J domain but leave the LxCxE motif intact will allow binding of Tag to the pRB family but drastically influence certain other effects of Tag on pRB proteins, therefore compromising the transformation potential of Tag. Although inactivating mutations in p107 and p130 have been rarely found in human tumors, their role in tumor suppression has been suggested by studies with SV40 Tag. Studies with mouse embryo fibroblasts (MEFs) lacking the retinoblastoma gene ($Rb^{-/-}$) have suggested that p130 and p107 may also be targeted by SV40 Tag to effect transformation. In $Rb^{-/-}$ MEFs, Tag with an intact LxCxE binds to p107 and p130 and disrupts their complexes with E2F. Further, Tag with an intact LxCxE confers a growth advantage in both $Rb^{+/+}$ and $Rb^{-/-}$ MEFs, suggesting a role of p107 and p130 in Tag-mediated transformation.[46] Tag has a novel effect on the phosphorylation state of p130 and p107 that differs from its effect on pRB. In cells expressing Tag, p107 and p130 are detected only in a hypo-phosphorylated form. Besides the LxCxE motif, integrity of the J domain of Tag is required for this effect, Moreover, Tag has been shown to promote degradation of p130, and the J domain appears to be essential for this effect as well. Further, the Tag J repression of E2F activity by p130 and pRB. One clue to the role of the J domain in these activities of Tag has been provided by recent work that demonstrated that Tag dissociates p130 from E2F-4, and the integrity of the J domain, hsc70, and ATP are required for this effect (Figure 1.5).[47,48] It is therefore possible that one function of the J domain is to couple

ATP hydrolysis by hsc70 to the endothermic disruption of the p130/E2F-4 complex. p53 made its debut as a cellular protein discovered in tight association with SV40 large Tag.[39,40]

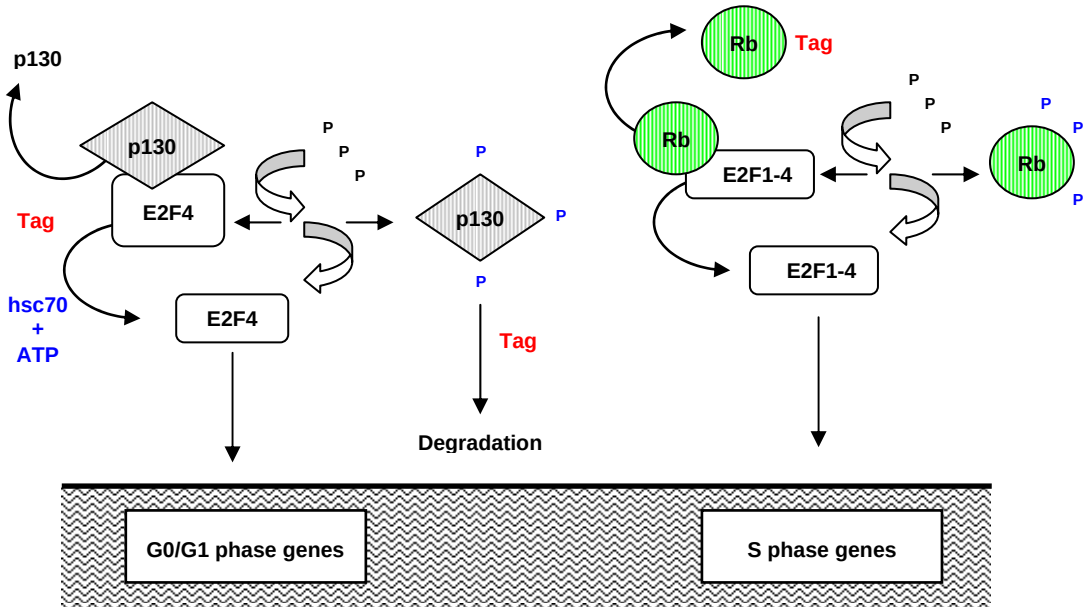


Figure 1.5 Manipulation of RB family members by Tag. Models of Tag-mediated disruption of the function of p130 and Rb, which are involved in the control, respectively, G0/G1 and S phase genes. Tag promotes p130 elemination, possibly through degradation of its hyperphoshorylated form. In addition, Tag disrupts the E2F4-p130 complex in a hsc70-ATP-dependent manner. Tag also sequesters the hyperphoshorylated form of Rb to relieve E2F transcriptional repression.

Since Tag, by then, had already claimed its status as one of the most powerful oncoproteins, p53 became an immediate focus of interest and was viewed as a clue in unravelling the mysteries of Tag-mediated transformation. In retrospect, the invested curiosity in p53 has generously paid off. During the past two decades, studies on p53 have given us a better understanding of the mechanism of cellular transformation by DNA tumor viruses, including SV40. On the other hand, as it became apparent that p53 was a tumor suppressor and the most commonly mutated gene in human cancers, work on p53 has led to an elucidation of the mechanisms of cell cycle control.[48] The tumor suppressor function of p53 is attributed to its negative regulation of cell proliferation. p53 brings about this repression, at least in part, by acting as a transcription factor.[49] DNA damage (due to, for instance, ionizing radiation) is an important stimulus that

triggers a p53 response. Following DNA damage, p53 levels in the cell increase with a corresponding increase in p53-mediated transcription. This increase in p53 levels results in cell cycle arrest followed by either DNA repair or, when DNA damage is irreparable, apoptosis. The p53-mediated arrest of cell proliferation operates in the G1/S and G2 phases of the cell cycle. In the G1/S phase, p53-mediated regulation is through transcriptional activation of genes encoding $p21^{Waf1/Cip1}$, bax, cyclin G1, mdm2, and several other factors. Perhaps the best understood of these is the regulation of p21 by p53. In G1, p21 negatively regulates the kinase activity of cyclin E/cdk2 and cyclin D/cdk4-6.[50] These kinases phosphorylate pRB and the related proteins p107 and p130 during G1. pRB proteins, in turn, regulate the E2F-mediated transcription required for cell cycle progression. In their hypophosphorylated state, pRB proteins bind to and repress E2F, thus arresting the cell cycle in G1. Therefore, in response to DNA damage, when p53 levels are upregulated and p21 expression is increased, the G1-phosphorylation of pRB proteins is inhibited. pRB remains bound to E2F, repressing E2F-mediated transcription, leading to cell cycle arrest (figure 1.6). G2 arrest by p53 is less clear at this point, but it may involve the effectors p21 and 14-3-3σ.[51] The functional significance of Tag-p53 interaction is not fully understood but the general postulation is that Tag inactivates the tumor suppressor function of p53. Tag can bind directly to the specific DNA binding domain of p53. Indeed, most inactivating mutations of p53 that disrupt DNA sequemce-specific binding by p53 also disrupt Tag binding. Work supporting this hypothesis demonstrates that Tag binding can interfere with the transcriptional activation of a reporter gene by p53.[52] It has been suggested that the oncogenic potential of both Tag and some mutant p53 proteins is the result of their ability to block binding of wild-type p53 to DNA and prevent transcription.[53] The control of p53 activity is also regulated by degradation. The best understood player in this pathway is mdm2. Not only does mdm2 inactivate the transcriptional activity of p53 by binding to and concealing its transactivation domain, but it also facilitates the ubiquitination-dependent degradation of p53, therefore controlling its stability. mdm2 expression is positively controlled by p53 through a feedback loop. There is evidence that mdm2 may require binding to p300 for p53 degradation. It has been demonstrated that the binding of p53 with Tag increases the half-life and steady-state levels of p53 co-precipitates in a trimeric complex containing p53 and Tag in vivo.[54,55] It is

conceivable, therefore, that the effect of Tag on enhancing the stability of p53 is partly due to the entrapment of the p300/mdm2/p53 complex that targets p53 for degradation. Since p53 sequestered by Tag is already transcriptionally inactive, the question that remains to be answered is why Tag concomitantly needs to prevent degradation of p53 by mdm2. Viral oncoproteins, including SV40 large Tag, cause simultaneous inactivation of both p53 and pRB to achieve cell transformation. In many human tumors, mutations in p53 are frequently observed in conjunction with mutations in Rb-1, suggesting cooperation between the two tumor suppressor. One point of convergence in the action of p53 and pRB is E2F-1. For instance, loss of pRB function leads to p53-mediated apoptosis, but forced overexpression of E2F-1 can override pRB function and induce p53-dependent apoptosis.[56] Evidence such as this indicates that E2F-1 may be a link between p53 and pRB pathways (Figure 1.6). p300 was first identified through its interaction with Ad E1A and has been shown to bind to SV40 large Tag as well. p300 has close structural and functional homology with CREB binding protein (CBP), which was first identified through its interaction with transcription factor CREB. Both p300 and CBP interact with a variety of cellular factors including p53, mdm2 and NFkB p65 subunit, implicating their involvement in multiple functions, and an influence on cell growth and transformation. There are also indications that p300 and CBP may have tumor suppressor function. Mice with a null mutation in one CBP allele develop hematological malignancies. p300 mutations have also been associated with various human carcinomas. Since p300 and CBP can each associate with p53, it is possible that their growth suppression is partly due to this interaction. Studies have shown that p53 interaction with p300/CBP contributes to p53-mediated transcriptional activation of genes (i.e. mdm2, p21 and bax) involved in DNA damage response, thereby contributing to p53-dependent G1 arrest and apoptosis. In addition to coordinating the p53 transactivation function, CBP/p300 also participate in the control of its metabolic stability. p300 interacts independently with both mdm2 and p53 and cooperates in mdm2-mediated degradation of p53. (Figure 1.6). There appears to be a contradiction, therefore, in the two effects of p300/CBP on p53 function promotion of transcriptional activation facilitates, whereas cooperation in mdm2-mediated degradation antagonizes, p53 function. It is possible, however, that these paradoxical effects of p300/CBP operate in different states of the cell cycle. When

p53 levels are low, p300/CBP helps in its transcriptional function, but when (following DNA damage, for instance) p53 levels are upregulated p300/CBP switches to the mdm2-involving path of p53 degradation. An early experment suggested that Tag functionally interacted with the p300 family. Expression of wild type Tag could complement a mutant of Ad E1A that was defective in p300 binding in a cellular transformation assay. Mutation of the N-terminal J-domain of Tag rendered Tag unable to complement a p300-binding defective mutant of E1A. In addition, an N-terminal mutant of Tag, T50-L7, lacking the residues 98-126, does not bind efficiently to p300/CBP and shows deficiency in its growth-stimulating activity compared to wild type Tag. It is not certain, however, where p300/CBP binds on Tag. More recent studies have suggested that Tag binding sites of p300 and CBP overlap with that of p53 (Figure 1.4), and Tag mutants defective in p53 binding also fail to bind p300, indicating that the association of the p300 family to Tag involves residues in the C terminus of Tag and may require p53 as an adapter.[57-60] Avantaggiati *et al*.[61] and Lill *et al*.[62] have shown that Tag interacts exclusively with the unphosphorylated form of p300. Since p300, like pRB, is differentially phosphorylated during cell cycle, preferential binding by Tag may have implications in Tag-mediated transformation. There is evidence suggesting that interactions of CBP/p300 with the E2F-1 transcription factor controls a variety of promoters involved in cell cycle progression. Trouche *et al*.[63] reported that the transcriptional activity of E2F-1 depends on its interaction with CBP. This group found that CBP increases E2F-1-mediated transcriptional activation. It is possible, therefore, that the ability of viral oncoproteins, such as E1A and Tag, to activate E2F-1 directed transcription is due to their ability to deliver CBP/p300 to E2F-1 responsive promoters, while at the same time removing pRB. A biochemical association between p300/CBP and E2F-1 has, however, not been demonstrated in vivo so far. Another aspect of binding of p300 with Tag was discussed earlier in terms of p53 stability. It is possible that the binding of p300/CBP by Tag has a dual role, one in the activation of E2F-1-mediated transcription and the other in the inhibition of p53 degradation (Figure 1.6).

<u>New proteins, new functions</u>

The enigma of Tag-mediated transformation continues to unfold. Some new functions of Tag have recently come to light. A third member of the p300/CBP family, namely, p400, was recently shown to be associated with Tag. Structurally, p400 is closely

related to p300 and CBP and, like its two relatives, is shown to interact with p53 and bind to AdE1A. Moreover, as in case of p300/CBP, the residues 501-550 of SV40 Tag appear to be involved in binding with p400 as well (Figure 1.4). The function of p400 is still unknown, but its homology with p300 and CBP, and its association with Tag, point to its significance in an important pathway waiting to be uncovered. A 185 kD protein that also co-precipitated with Tag was identified in 1992, which may be the same as a recently described 193 kD Tag-associated protein.[64]

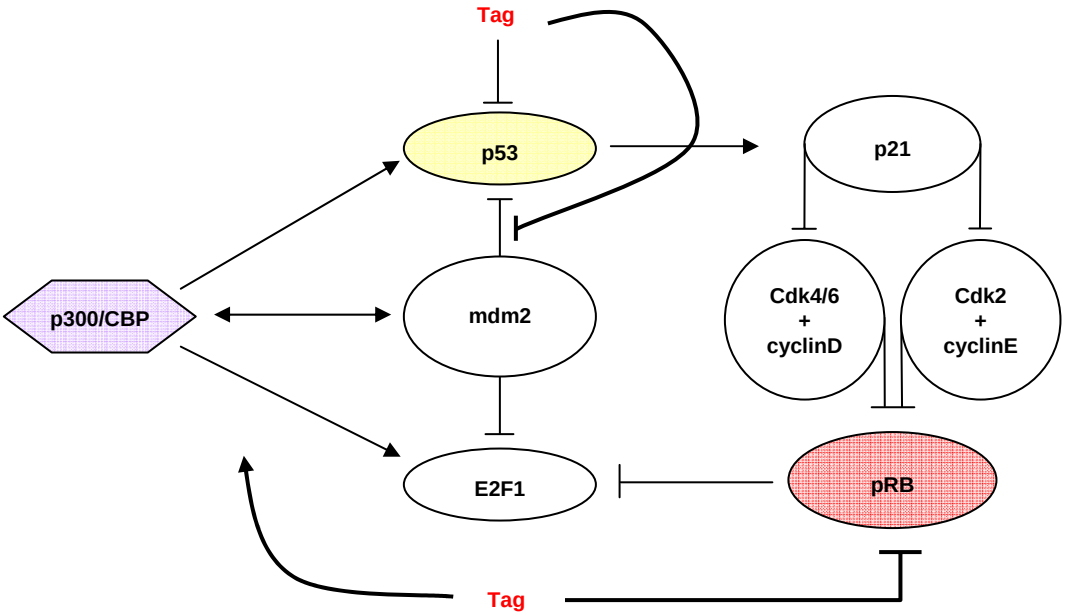


Figure 1.6 Disruption of p53 and p300/CBP by TAg. p53 can stimulate p21 expression which, through inactivation of Rb phosphorylating kinases, can inhibit E2F1-mediated transcription, leading to cell cycle arrest. Steady-state levels of p53 are regulated by mdm2, an E3 ligase that catalyzes ubiquitination-degradation of p53. E2F1-mediated transcription and mdm2 degradation activity are also regulated by p300/CBP. In the model depicted here, TAg binds to both p53 and p300/CBP, thus manipulating the activity of E2F1 as well as of mdm2, through multiple routes. In addition, TAg binds to and disrupts the functions of another important player of this pathway, i.e. pRB.

This protein has a bcl-2 homology that enables it to promote apoptosis when transiently overexpressed in cells. The pro-apoptotic function of this protein was shown to be abolished by Tag expression. This novel protein, therefore, opens yet another window on the Tag-mediated transformation scenario. Another recently reported novel function of Tag is an antiapoptotic activity that is independent of Tag-mediated inactivation of p53. This antiapoptotic activity of Tag appears to reside in its residues 525-541, which bear homology with the bcl-2 family's BH-1 domain, a region associated with

protection from apoptosis. New functions of SV40 large T continue to surface. On the other hand, cellular pathways already known to be deregulated by Tag await further elucidation. The study of Tag-mediated transformation therefore holds further promise of a better understanding of the cell cycle, and of its disruption that leads to carcinogenesis.[65]

1.3.2 SV40 small T antigen and PP2A phosphatase in cell transformation

SV40 tag exhibits an all α-helix structure with two zinc-binding sites in the unique domain (Figure 1.7). The J domain contains three helices and has a structure similar to the previously solved crystal structure of the J domain of SV40 Tag and a NMR structure of polyomavirus DnaJ-like domain, with Cα root mean square deviations (RMSDs) of 1.84 and 1.83 Å, respectively.[66]

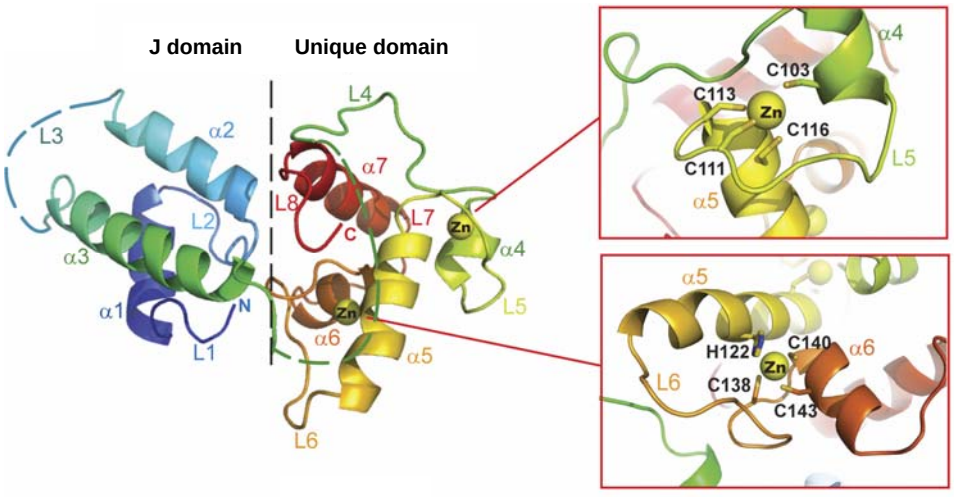


Figure 1.7 Structure of SV40 tag. Structural organization of SV40 tag and the zinc coordination of tag unique domain. The peptide chain is color-coded from blue to red, going through the rainbow colors, from the N terminus (blue) to the C terminus (red).

The contribution of tag to human cell transformation derives directly from the inactivation of the pRB and p53 pathways, while the best-studied function of tag is its ability to inhibit PP2A activity. Examination of tag mutants has provided important clues to the functions of tag during cell transformation. Mutations in tag that prevent the interaction with and inhibition of PP2A demonstrate that this interaction is

required for tag-mediated transformation. Conversely, a tag mutant that contains only the PP2A-inactivation domain (amino acids 88–174) retains the ability to induce transformation. These observations suggest that the perturbation of PP2A activity is necessary for the transforming activity of tag.[67,68]

PP2A is a multi-complex enzyme

Protein phosphatase 2A (PP2A) is a ubiquitously expressed protein serine–threonine phosphatase that is implicated in the regulation of numerous signaling pathways. The term PP2A refers to a diverse family of phosphatases containing a common catalytic C subunit whose activity is regulated by a diverse set of regulatory proteins. The most common forms of PP2A contain a highly active core dimer composed of a catalytic C subunit, also called PP2AC or PPP2C, and a scaffolding A subunit, also known as PR65 or PPP2R1. The scaffolding subunit mediates formation of heterotrimeric holoenzymes by binding additional regulatory B subunits that dictate the substrate specificity, localization and functions of the specific complexes. Each of these subunits exists in multiple different isoforms and splicing variants that form distinct ABC holoenzyme complexes (Figure 1.8). In mammals, the PP2A A and C subunits are each encoded by two different genes. The two isoforms of the C subunit, Cα and Cβ, share 97% identity, with only eight amino acid differences. Despite this degree of identity, these forms have non-redundant functions. The two PP2A A subunit isoforms (also called PR65 or PPP2R1, Aα and Aβ) are 87% identical, but the Aα isoform is approximately tenfold more abundant than the Aβ isoform in most tissues. Detailed mutagenesis and structural studies have suggested that the free A subunit is a rod-shaped molecule consisting of 15 non-identical HEAT repeats (named for its presence in huntingtin, elongation factor 3, PP2A A subunit, and the TOR kinase). The amino-terminal HEAT repeats 1–10 are responsible for binding regulatory subunit, while the carboxy-terminal HEAT repeats 11–15 bind to the C subunit. While the A subunit primarily serves a structural role, specific single amino acid alterations in either of the A subunits disrupts the binding of specific B subunits, suggesting that the A subunits regulate PP2A holoenzyme composition.[69-71] Four unrelated families of B subunits have been identified to date, and several different nomenclature systems are present in the literature. The B family, also referred to as B55, PR55, or PPP2R2, consists of α, β, γ, and δ isoforms. The B' family, known as well as B56, PR61, or PPP2R5, contains α, β, γ, δ and ε isoforms.

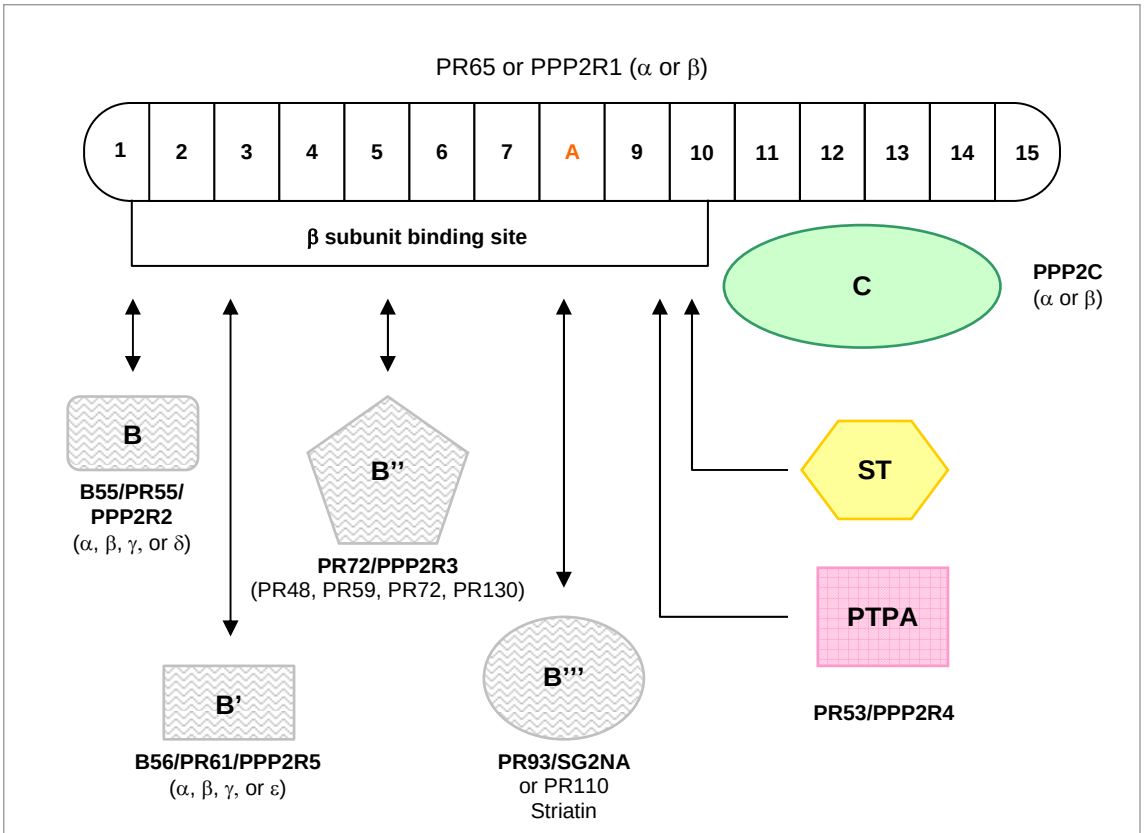


Figure 1.8 PP2A holoenzyme composition. The PP2A A subunit (PR65 or PPP2R1) exists in mammals as Aα and Aβ isoforms. Both isoforms consist of 15 HEAT repeats (indicated as numbered sections of PP2A A) that mediate binding of the regulatory B and catalytic C subunits. The C subunit (PP2AC or PPP2C1) binds to HEAT repeats 11–15 and also exists as two isoforms, Cα and Cβ. Five classes of cellular B subunits have been identified to date, and a single B subunit binds to HEAT repeats 1–10 to complete the complex. The B family (also the B55, PR55, or PPP2R2 family) subunits are encoded by four genes (*enclosed in parentheses*), while five genes encode subunits of the B′ family (also the B56, PR61, or PPP2R5 family). The B'' family (also the PR72 or PPP2R3 family) consists of four isoforms encoded by three genes. Two calmodulin-binding proteins comprise the B''' family. PTPA (also PR53 or PPP2R4) is a PP2A phosphotyrosyl phosphatase activator protein. In addition, SV40 ST interacts with the B subunit-binding region of the PP2A A subunit, resulting in the displacement of the B subunits from the PP2A complex.

Within both of these families, some isoforms exist as multiple variants encoded by alternative splicing. The B'' family, also named PR72 or PPP2R3, is comprised of the PR48/PR70, PR59, PR72, and PR130 isoforms, the last two of which are alternatively spliced from a single gene. A fourth family, called B''', has been identified and consists of the calmodulin-binding proteins PR93/SG2NA and PR110/Striatin. Considering alternative transcripts, over 20 B subunits have been identified. While the A and C isoforms are expressed in most of cell types, some of the B subunits show tissue- and developmental stage-specific expression patterns. An enzymatically active PP2A related heterodimer complex can also be formed through the interactions of the C

subunit and Tap42/α4 in place of the structural A subunit.[72-74] Expression Tap42/α4 subunit is required to repress apoptosis in mammalian cells through negative regulation of two transcription factors, c-Jun and p53. In addition to its serine–threonine phosphatase activity, PP2A exhibits a basal level of phosphotyrosyl phosphatase activity, which is stimulated by interaction of the AC dimer with Phosphotyrosyl Phosphatase activator, also known as PP2A phosphatase activator, PTPA or PPP2R4. tag modulates the substrate specificity and phosphatase activity of the AC core complex through the direct binding to the scaffolding A subunit. Recent structure analyses revealed that tag interacts with the HEAT repeats 3–7 of the A subunit, which overlaps with the binding site for the PP2A B regulatory subunits. The ability of tag to displace multiple B subunits from the complex A subunit demonstrated both *in vitro* and *in vivo*. However, in recent report tag was found to have a lower binding affinity than B56γ for the PP2A core enzyme *in vitro*, suggesting that tag may preferentially disassemble PP2A complexes containing specific regulatory subunits. The interplay between tag and the PP2A complexes may be even more complicated *in vivo*. Tag may directly associates with newly synthesized AC core dimers, perturbing access of B subunits to the C subunit. Since individual PP2A monomeric subunits are quickly degraded, binding of tag to the AC dimers may down-regulate *de novo* synthesis of certain PP2A B regulatory subunits. Indeed, tag expression in monkey kidney CV-1 and epithelial MDCK cells induces the down-regulation of PP2A Bα subunit. However, additional studies are required to determine how tag expression affects the localization and activity of particular PP2A complexes. Nevertheless, inhibition of PP2A complexes by tag alters multiple pathways involved in cell survival, growth, apoptosis, and adhesion and induces transformation.[75-78]

tag promotes cell proliferation

The observation that tag expression results in enhanced proliferation rates suggests the involvement of tag in the regulation of cell cycle progression. Specifically, tag promotes the transition from G1 to S phase. Several studies have implicated tag in regulating the mitogen activated protein kinase (MAPK) cascade at several discrete points, such as Raf, MEK1/2, ERK1/2, and KSR. Activation of the MAPK cascade induced by tag overexpression leads to up-regulation of AP-1 transcriptional activity. Tag also stabilizes c-Myc and enhances its transcriptional activity. Specifically, PP2A

holoenzymes containing the B56α regulatory subunit were found to associate with and directly dephosphorylate c-Myc at Ser62, a residue previously implicated in targeting c-Myc to proteosome degradation. Notably, a stabilized c-Myc mutant can substitute for tag expression in cell culture transformation assays.[79] Finally, several other transcriptional factors such as CREB (cAMP regulatory element binding protein), Sp-1, and E2F transactivation of which is linked to cell cycle progression, are also known to be activated by tag. As a consequence of tag expression, the expression of several proteins associated with cell cycle progression such cyclin D1, cyclin B, thymidine kinase, dihydrofolate reductase are elevated. However, further work is necessary to determine the specific PP2A subunits that regulate particular aspects of cell proliferation.[80,81]

S2 effects of tag on anti-apoptotic pathways

Several lines of evidence implicate tag in regulation of apoptosis. Transcriptional analysis of human cells expressing tag revealed up-regulation of anti-apoptotic targets of NF-kB such as *ALDH1*, *SERPINB2*, *SERPINB2* and *surviving*. tag expression appears to activate NF-kB via protein kinase C and phosphatidylinositol 3-kinase (PI3K) signaling. Furthermore, PP2A inactivates the IκB kinase, a positive regulator of NF-κB signaling. tag has also been implicated in the regulation of the PI3K/Akt pathway. In transformation experiments, either an activated allele of PI3K or a combination of the activated PI3K effectors Akt and Rac1 can replace tag and induce human cell transformation. Conversely, blocking PI3K function inhibited ST-mediated transformation. Consistent with these results, both the treatment of cells with the PP2A inhibitor okadaic acid and expression of tag induces phosphorylation and activation of Akt. Since PP2A was found to bind to p70S6K *in vivo* and inhibit its activity *in vitro*, PP2A complexes may also act downstream of Akt in the mTOR pathway through regulation of the mTOR substrate p70S6K. These observations also explain the ability of tag to enhance cell proliferation in low-nutrient conditions.[82,83]

tag induces changes in the cytoskeleton

tag expression also targets PP2A-dependent processes governing the cytoskeleton. Overexpression of tag in epithelial cells induces dramatic F-actin rearrangements, including increased Rac1-induced membrane ruffling, Cdc42-initiated filopodia and loss of RhoA-dependent stress fibers. Transcriptional profiling analyses have also

revealed that a large number of genes involved in cellular adhesion and motility such as *osteopontin*, *paxillin* and *gelsolin* are up-regulated by the presence of tag. The role of SV40 in the regulation of β1 integrin-F-actin anchoring at focal adhesion sites was further confirmed by the finding that the PP2A AC core dimer forms complexes with Rac1, β1-integrin, and the actin cross-linking protein, IQGAP1. In contrast to the apparent activation of integrin signaling, tag causes down-regulation of many genes important for cell–cell adhesion and junctional adhesion such as *ICAM-1*, *VCAM-1*, *plakoglobin* and *claudin 11*. Consistent with these observations, severe defects in the formation and barrier properties of tight junctions are observed in cells that express tag (Figure 1.9).[84,85]

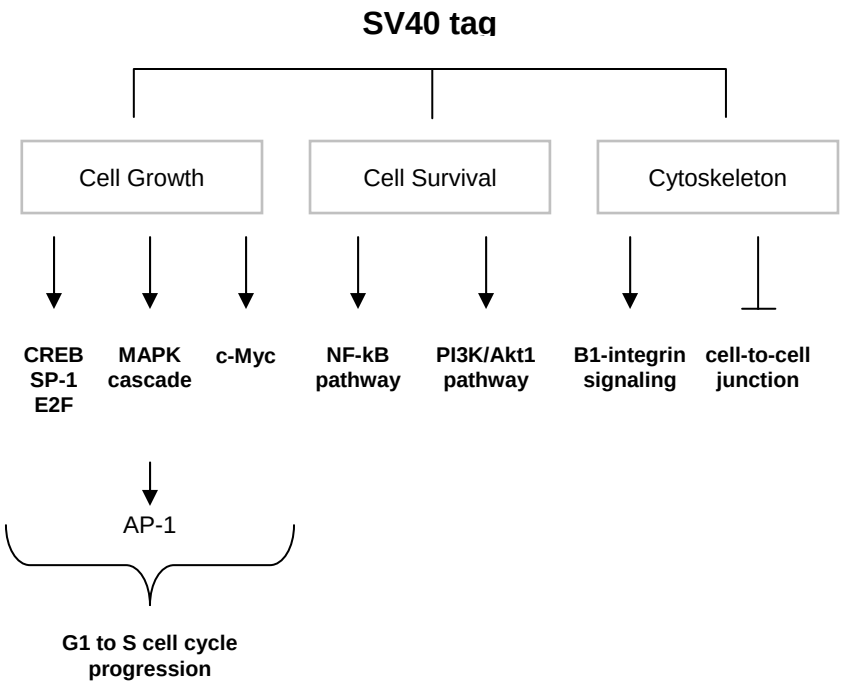


Figure 1.9 Downstream effects of tag expression relevant to cell transformation. By inhibiting multiple PP2A complexes tag activates numerous signaling pathways that may converge to facilitate cell transformation. This results in increased proliferation, enhanced survival rate, and cytoskeletal changes. Studies to date reveal that the stabilization of c-Myc and/or activation of Akt can contribute to cell transformation mediated by tag (*in italics*) while additional work is required to determine which of the other pathways shown here are directly involved in tag-mediated transformation

Alterations of PP2A structural subunits in cancer

Several laboratories have identified mutations in PP2A structural subunits in spontaneously arising human cancers. Of the various PP2A subunits, alterations

involving the *PPP2R1B* gene encoding the Aβ subunit are the most commonly found. Specifically, somatic alterations of the gene encoding the PP2A Aβ structural subunit have been identified in 8–15% of colon cancers, 15% of lung cancers, 13% of breast cancers, and 6% of tumor cell lines. Reduced expression of the PP2A Aβ subunit also has been found in 16 of 32 cancer cell lines derived from human lung, colon, breast, and cervical carcinomas and from primary glioblastomas. Mutations of the more abundant PP2A Aα subunit have also been observed, although at a low frequency.[86] In addition to mutations, decreased expression of Aα subunit has been found in 25 out of 58 brain tumors and in the MCF-7 breast cancer cell line. The most common type of PP2A Aα and Aβ cancer-derived mutations are nucleotide substitutions that change highly conserved amino acids. The crystal structure of the PP2A complex predicts that these particular amino acids are involved in the formation of heterotrimeric complexes, suggesting that cancer-derived mutations disrupt PP2A holoenzymes. Indeed, each of the reported PP2A Aα and Aβ mutants are defective in binding to the B and/or C subunits and exhibit impaired phosphatase activity. Transformation assays also confirm that mutations affecting PP2A structural isoforms form functionally null alleles. These alterations of PP2A structural subunits suggest that PP2A is inactivated during cancer progression while PP2A dysfunction may contribute to spontaneously arising human cancers.[87]

The tumor suppressive properties of PP2A Aα subunit

Analysis of PP2A Aα mutations in cancer samples revealed that PP2A Aα mutations predominantly involve only a single allele. Indeed, complete loss of PP2A in Drosophila, mouse and human cells induces cell death by apoptosis. Reduction of PP2A Aα expression by approximately 50% conferred a tumorigenic phenotype. Overexpression of wild type PP2A Aα inhibited cell transformation induced by its partial suppression while cancer-associated PP2A Aα mutants failed to reverse the transforming phenotype. These observations suggest that cancer-associated PP2A Aα mutations contribute to human cell transformation by creating a state of haploinsufficiency. PP2A Aα mutants are defective in binding to all B56 family members. Thus, a mutation in one Aα allele effectively decreases by half the amount of Aα available for PP2A B56 subunit binding. In human cells, suppression of Aα expression by 50% leads to complete loss of AαC–B56γ heterotrimeric complexes. The

AαC–B56γ complexes are of particular interest since suppression of PP2A B56γ regulatory subunit was previously shown to cooperate with the expression of LT, hTERT and H-Ras to induce transformation. Moreover, overexpression of PP2A B56γ3 isoform in lung cancer cell lines at least partially reverses the tumorigenic phenotype of these cells. Together these observations suggest that haploinsufficiency of PP2A leads to selective loss of the B56γ-containing PP2A complexes. At present, conflicting data have been reported concerning expression levels of this subunit in human cancers. While one group found higher expression levels of the PP2A B56γ mRNA in human melanoma cell lines compared to normal melanocytes, a second group observed decreased expression in primary human melanoma samples when compared to melanocytic nevi. Consistent with the data from the second group, analysis of a sample of ten lung cancer cell lines failed to detect any B56γ at the protein level. Thus while some data suggests that loss of B56γ contributes to cancer development, further work is necessary to determine whether B56γ is truly a tumor suppressor gene. The specific substrates of AαC–B56γ complexes are not fully understood. Both B56γ and Aα dysfunction lead to activation of the Akt pathway, strongly implicating activation of the Akt pathway in human cell transformation induced by loss of AαC–B56γ complexes. Consistent with this idea, either activated PI3K or a combination of the activated PI3K effectors Akt and Rac1 can substitute for ST and induce human cell transformation. B56γ may also contribute to tumor development through regulation of the two well-known tumor suppressors, APC and/or p53. Both APC and p53 directly interact with B56γ regulatory subunit. By binding to APC, PP2A B56γ inhibits formation of APC-axin complexes leading destabilization of the β-catenin protein. As result, overexpression of B56γ reduces the abundance of β-catenin and inhibits transcription of β-catenin target genes. PP2A B56γ complexes may regulate p53 by at least two distinct mechanisms. PP2A B56γ1 and B56γ3-containing complexes can directly mediate dephosphorylation of p53 at Thr55 that prevents its proteasome-mediated degradation. Dephosphorylation of p53 at Thr55 is required for B56γ3-mediated inhibition of cell proliferation and transformation. Moreover, a p53 target gene, cyclin G, recruits PP2A B56γ and modulates the phosphorylation of Mdm2. Inhibition of Mdm2 by PP2A B56γ–cyclin G complexes results in stabilization of p53. However, while these data imply that inactivation of PP2A complexes perturbs p53 activation, this effect is not

sufficient to transform human cells in the absence of direct inhibition of p53 by Tag or other means. In addition, it remains unclear whether activation of the β-catenin pathway suffices to substitute ST in cell transformation models.[88-90]

The role of PP2A Aβ in tumor suppression

In contrast to Aα, mutations in one allele of the other PP2A structural subunit Aβ is usually accompanied by deletion or mutation of the second allele resulting in disruption of both alleles. Experimentally, loss-of-function of both Aβ alleles cooperates with the expression of Tag, hTERT and H-Ras to convert human cells to transformed phenotype. Moreover, introduction of a wild type Aβ allele into lung cancer cell lines carrying loss-of-function Aβ mutations partially reversed the tumorigenic phenotype of these cells. Together these observations implicate PP2A Aβ as a tumor suppressor gene. Although PP2A Aα and Aβ show considerable homology (86%), they are surprisingly different functionally. Overexpression of Aα is not able to reverse the transforming phenotype mediated by Aβ suppression, suggesting that dysfunction of PP2A Aα and Aβ contributes to cell transformation by discrete mechanisms (Figure 1.10). Proteomic screening for PP2A Aβ specific substrates revealed that PP2A Aβ dephosphorylates the Ras family member RalA GTPase at Ser183 and Ser 194. The RalA dephosphorylation resulted in dramatic decrease of RalA activity as detected by its ability to bind the RalBP-1 effector protein. The RalA GTPase is implicated in regulation of several signaling pathways relevant to transformation. Pathways reported to be regulated by RalA include the activation of phospholipase D1 and Src kinase, vesicle transport, increased cell motility and anchorage-independent growth. Loss of function experiments revealed that RalA is crucial for transformation mediated by Aβ dysfunction. This observation together with the finding that phosphorylation of RalA affects its transforming activity suggest that accumulation of phospho-RalA in PP2A Aβ-deficient cells promotes cell transformation. The downstream events of RalA-mediated transformation remain undefined, since activation of known RalA effector proteins such as RalBP1 or the exocyst proteins has not been directly linked to tumorigenesis. Other substrates of PP2A Aβ complexes may also contribute to cell transformation and have yet to be identified.[91]

Other mechanisms of PP2A inactivation during cancer progression

In addition to viral oncoproteins, other potentially oncogenic proteins may also affect

PP2A function. The phosphoprotein SET has been identified as a specific inhibitor of PP2A. SET is a regulator of various physiological processes including histone acetylation, regulation of transcription, cell growth and transformation.

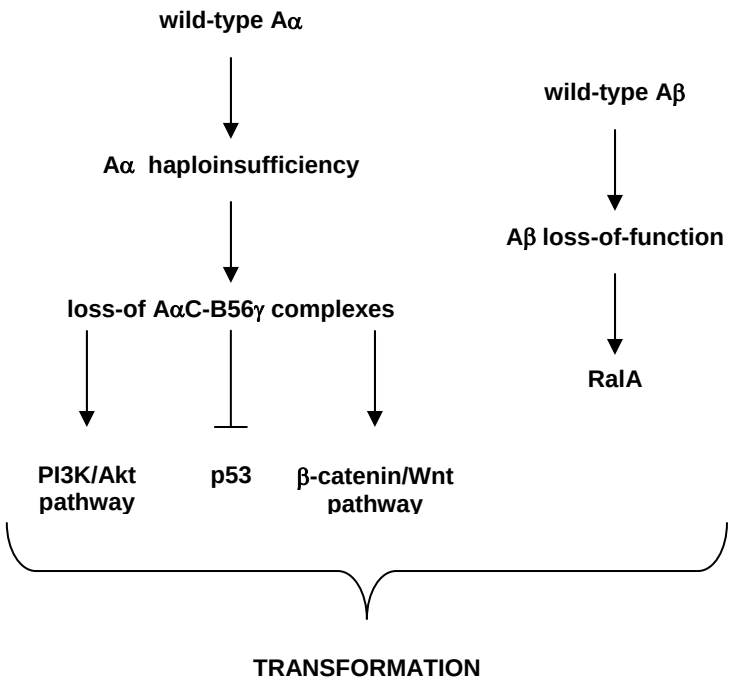


Figure 1.10 Inactivation of the structural PP2A Aα and Aβ subunits contribute to cell transformation by distinct mechanisms. Cancer-associated PP2A Aα mutations result in Aα haploinsufficiency, which may induce human cell transformation by selectively eliminating PP2A B56γ containing complexes. Loss of B56γ-containing complexes results in constitutive AKT phosphorylation, accumulation of β-catenin, and p53 degradation. In contrast, PP2A Aβ loss-of-function permits the accumulation of activated RalA

SET upregulation has been observed in chronic myelogenous leukemia and Wilm's tumors. Moreover, SET was found to be a fusion partner with the CAN/Nup214 gene in an acute undifferentiated leukemia fusion gene. It has been reported that both PP2A and SET interact with the HRX leukemic fusion protein in myeloid cell extracts and that these complexes participate in the deregulation of cell proliferation and differentiation in leukemia. However, SET also regulates the tumor suppressor Nm23-H1, a granzyme A-activated DNase. Thus, further studies are required to assess interconnection between SET and PP2A during cancer progression and to determine the contribution of PP2A to SET-dependent cellular processes. The recently identified Cancerous Inhibitor of PP2A (CIP2A) was found to inhibit PP2A activity towards

c-Myc thereby preventing its proteolytic degradation. Suppression of CIP2A inhibits anchorage independent cell growth and tumor growth *in vivo* while CIP2A overexpression cooperates with Ras and c-Myc oncoproteins to induce tumorigenic conversion of mouse primary embryo fibroblasts. Expression of CIP2A was found to be up-regulated in head and neck squamous cell carcinomas and in colon cancer. However, c-Myc stabilization only partially explains the observed phenotype caused by CIP2 overexpression, indicating that inhibition of other PP2A specific functions by CIP2 can also contribute to cell transformation.[92,93]

1.4 SV40 life cycle in host cells

The cell infection starts by the binding of the SV40 virus to a receptor on the cell membrane. This receptor has been identified as the major histocompatibility complex (MHC). In monkeys, initial lytic infection by SV40 is controlled by the immune system. Then, SV40 persisting infection occurs in the kidney cells where it may be reactivated by immunosuppression. The life cycle of SV40 in humans is poorly understood. After binding to the cell surface, polyomavirus capsid undergoes endocytosis and is transported to the nucleus where the viral DNA is uncoated and transcription of the early region begins. SV40 enters cells by caveola-mediated endocytosis. The primary transcript from the early region is alternatively spliced to give two mRNAs that encode Tag and tag. Tag is a nuclear phosphoprotein of 94 kD and it is an essential factor for viral DNA replication. It binds to the viral origin of replication (ori) where it promotes unwinding of the double helix and recruitment of cellular proteins that are required for DNA synthesis, including DNA polymerase-α and replication protein A. SV40 relies on cellular enzymes and cofactors for DNA replication and these proteins are expressed in S phase. Tag modulates cellular signaling pathways to induce cells to enter S phase and this accounts for the ability of Tag to transform cells. Tag is thought to stimulate the cell cycle through its ability to bind to several cellular proteins that are involved in crucial signal transduction pathways that control cell cycle progression and apoptosis.[94] The role of the tag in the polyomavirus life cycle is less clear (Figure 1.11). Analysis of SV40 deletion mutants revealed that tag is not essential for lytic infection in culture. However, tag cooperates with Tag in the transformation of cells by SV40 and increases virus yield in permissive cells. As viral replication proceeds, the late genes

begin to be expressed. In permissive cells, Tag acts to stimulate transcription from the late promoter and represses transcription from the early promoter. The gene products of the late region are the capsid proteins VP1, VP2, and VP3, which assemble with the replicated viral DNA to form virions, which are released upon cell lysis. SV40 DNA can become integrated into the chromosomal DNA of the cell especially upon infection of non-permissive cells.[95]

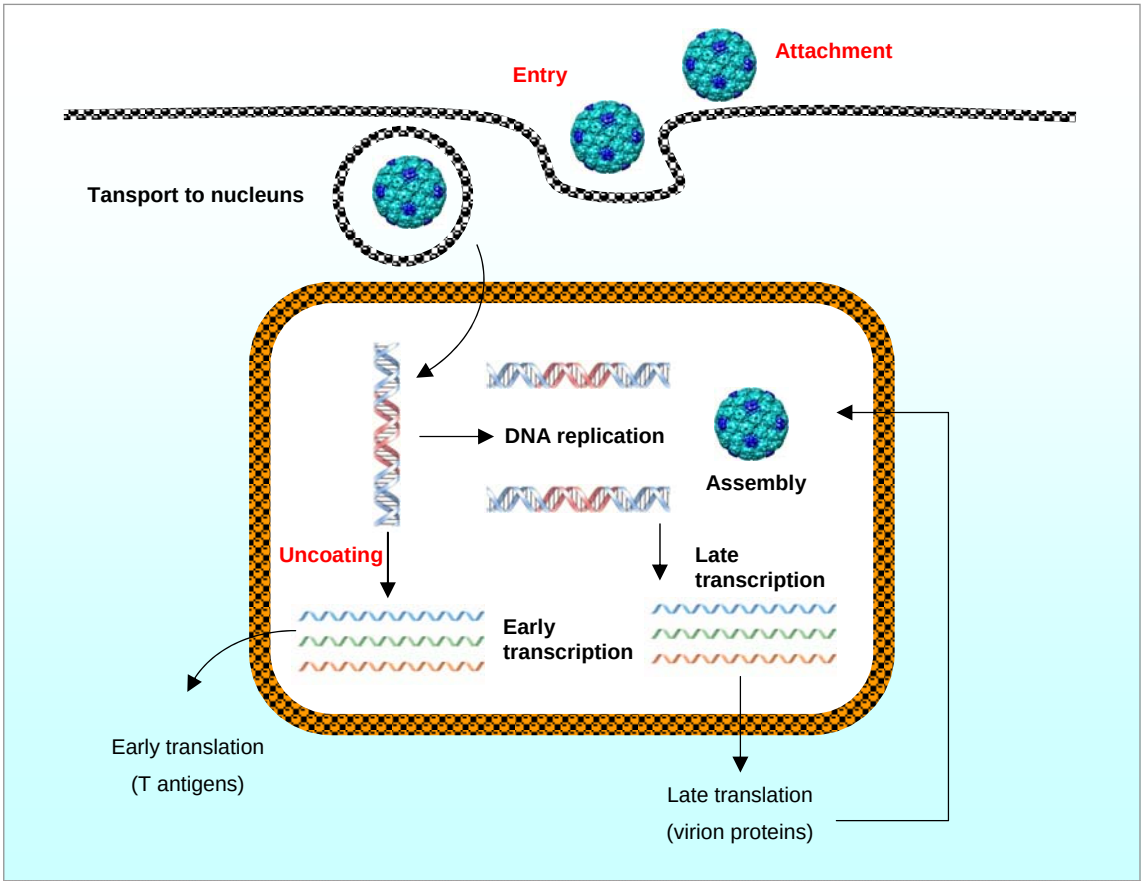


Figure 1.11 (a) Attachment:SV40 receptor appears to be MHC class I antigen VP1 (only external protein on virus capsid) responsible for receptor binding (anti-VP1 Abs block binding). **(b) Entry:**VP2/3 are myristylated & believed to interact with cellular membranes to facilitate entry. Virions are taken up by endocytosis and is transported to the nucleus by interaction of endocytic vacuoles with the cytoskeleton. **(c) Uncoating:** virus particles enter by the nuclear pores and uncoating occurs inside the nucleus.

Integration occurs at random both in terms of the site in the cellular genome and in the viral genome. Different studies indicate that SV40 can replicate productively in human cells, including spongioblasts, fetal neural cells, newborn kidney cells, and some tumor cell lines, although it grows poorly in human fibroblasts.[96] Some human cell types undergo visible cell lysis in response to SV40 infection, whereas other cells fail to

exhibit cytopathic changes and produce low virus levels. Human mesothelial cells do not support the lytic infection efficiently and are transformed at a high rate (1,000 times higher than that of human fibroblasts) by SV40 or Tag alone, and release SV40 virions as a result of the persistent infection. The same behavior has been observed in human lymphoblastoid B-cell lines where SV40 progeny is produced at a low level.[311] This behavior peculiar to SV40 in specific human cells, such as mesothelial cells, is uncommon for a DNA tumor virus. Indeed, it is well established that DNA tumor viruses either infect permissive cell lines with the production of an infectious viral progeny or infect and transform non-permissive cells without a productive viral cycle (Figure 1.11).[97]

1.5 Comparisons of the SV40, JCV, and BKV

SV40 is phylogenetically closely related to the human JCV and BKV. They evidence similarity with respect to size (~5.2 kb), genome organization and DNA sequence. The Tags of SV40, BKV and JCV strongly cross-react with the same antisera, while a less strong cross-reactivity is observed in most structural antigenic determinants of the viral proteins, named VP1, VP2 and VP3. A genus-specific capsid antigen, located on viral peptide VP1, has been identified. The DNA sequences of SV40 share 70% homology with BKV, and 69% with JCV. The greatest homology is found in the early region coding for the Tags and tags, whereas a lower homology is detected in the regulatory region (Figure 1.12).[98,99]

1.6 The immune response to SV40, JCV, and BKV

The main effector in the cellular immune response to viruses is the $CD8^+$ cytotoxic T lymphocyte (CTL), which lyses virus-infected cells following recognition of virus-encoded epitopes presented on the cell surface by major histocompatibility complex (MHC) class I molecules. Multiple factors contribute to successful $CD8^+$ T-cell recognition of virus epitopes, including the proper generation of the virus epitopes, successful loading and stable binding of virus epitopes to MHC class I molecules, proper trafficking of the complex to the cell surface, and the presence of T cells in the host repertoire capable of recognizing the virus epitopes. MHC class I molecules are

composed of a transmembrane heavy chain that associates noncovalently with β2-microglobulin and a bound peptide representing the T-cell-recognized epitope. The epitopes recognized by $CD8^+$ T cells are typically peptides of 8-10 amino acids in length that can be derived from a variety of virus proteins.

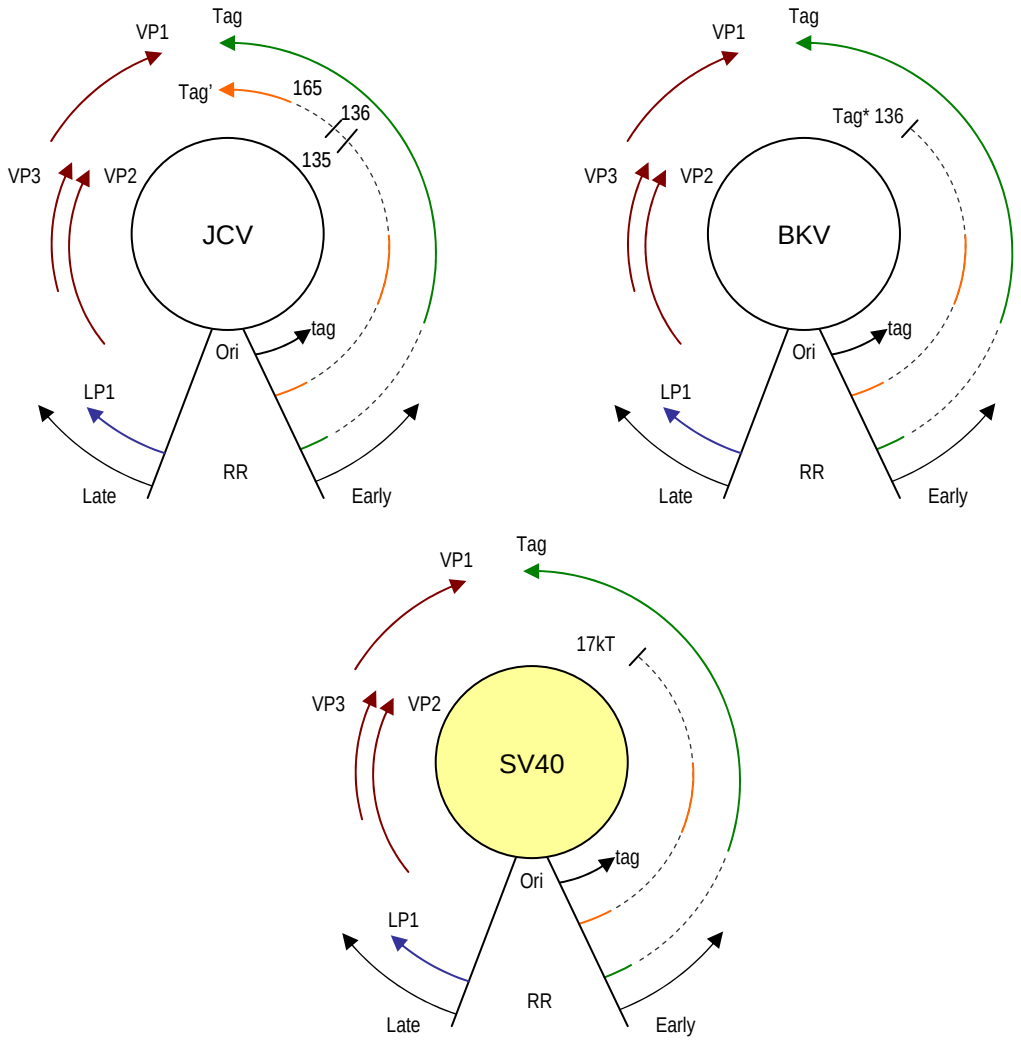


Figure 1.12 Comparisons of the JCV, BKV, and SV40 genomes. The organizations of these primate polyomavirus genomes are nearly identical. The inner circles represent the double-strended DNA genomes (5130 bp), JCV [Mad-1]; 5098 bp, BKV [ASI]; 5243 SV40 [776], and the outer arcs denote the encoded viral proteins. The genomes are divided into three regions. The early region specifies five (JCV) or three (BKV, SV40) regulatory proteins produced by translation of alternatively spliced early mRNAs. Tag', Tag*, and 17kT on the JCV, BKV, and SV40 maps, respectively, represent proteins encoded by mRNAs composed of three exons that may or may not be shared with the Tag transcript. Each genome may encode an early leader peptide (JELP, BELP, or SELP) of unknown function. The late region specifies four proteins required for capsid assembly, VP1-3 and LP1. The regulatory region (RR) contains the cis-acting elements that control viral DNA replication (Ori) and transcription. The promoter/enhancer signals for transcription are the least conserved sequences in the three genomes.

This length requirement is imposed by the structure of the peptide binding groove in which the closed ends of the groove accommodate the amino and carboxy termini of the peptide. The interactions between conserved residues of the MHC molecule and the free amino and carboxy termini of the peptide serve to stabilize peptide binding. The specificity of peptide binding to MHC molecules, however, is determined by the peptide sequence. Peptides that bind to a given MHC molecule typically contain two or three "anchor" residues that contribute to successful binding within the peptide binding groove. The side chains of these anchor residues are oriented into the peptide binding groove, where they are positioned into chemically and structurally compatible regions of the groove. Thus, peptides that bind to a particular MHC molecule generally share a sequence motif of anchor residues. Peptides are liberated from virus protein in the cytosol, most likely through the activity of a large cytosolic protease called the proteasome. This multisubunit, multicatalytic protease has been implicated in the generation of $CD8^+$ T-cell-recognized epitopes through the use of specific inhibitors as well as by the finding that some proteasome subunits are encoded within the MHC region of the chromosome, and their association with the proteasome can modify its activity. In particular, the activity of the proteasome leads to the liberation of peptides containing hydrophobic residues at the carboxy terminus, which typically serve as one of the anchor residues for binding to MHC class I molecules. Peptides are actively transported from the cytosol to the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP), which also has a preference for peptides containing a hydrophobic residue at the carboxy terminus. Once inside the ER, peptides are loaded onto partially folded MHC class I molecules. If peptides are not of optimal length for stable binding to MHC class I, some trimming can occur in the ER. Once a stable complex is formed, the folded complex is released from the ER and transits to the cell surface where it can be recognized by T cells specific for the particular epitope bound. Although a large number of peptides might be liberated from virus proteins, only those that from stable complexes with MHC class I are presented at the cell surface.[100,101] The stability of peptide/MHC class I complexes also contributes to the number of specific complexes found at the cell surface. Differences in stability con influence the hierarchy of $CD8^+$ T-cell responses in instances where multiple virus epitopes are presented for T-cell recognition.[102] Finally, the $CD8^+$ T-cell response of the

host will be limited by the T-cell receptors available in the host's repertoire. Thus, even though multiple virus-derived peptides might be presented at the cell surface for T-cell recognition, the host might contain T cells that can respond to only a few of these epitopes. Helper, or $CD4^+$, T cells recognize peptide antigens presented by MHC class II molecules expressed on specialized cells of the immune system, such as B lymphocytes and professional antigen-presenting cells (APCs). In contrast to antigens that enter the MHC class I processing pathway, exogenous antigens are generally processed for presentation by MHC class II molecules in endosomal vesicles. MCH class II molecules are composed of two transmembrane proteins, the a and b chains, which adopt a three-dimensional structure similar to that of MHC class I molecules. Importantly, the ands of the peptide binding groove of MHC class II molecules are more open than those observed for MHC class I molecules. This usurps the length restriction imposed on MHC class I binding peptides such that peptides of varying lengths can bind in the groove of MHC class II molecules. Consequently, the stability of peptide binding to MHC class II molecules is maintained through interactions with the main chain atoms of the peptide. Although less well defined than for MHC class I binding peptides, MHC class II binding peptides also contain anchor residues for binding to a particular MHC class II molecule. MHC class II molecules are co-translationally inserted into the ER but do not bind to peptides in the ER due to interaction with a chaperone molecule called the invariant chain (Ic) that blocks access to the peptide binding groove of MHC class II molecules. In addition, Ic targets MHC class II molecules to the endosomal/lysosomal pathway in which limited proteolysis degrades the major portion of Ic, leaving a peptide fragment, called CLIP, which remains bound in the peptide binding groove. Another molecules encoded within the MHC region, called HLA-DM, mediates the exchange of CLIP for peptides generated from proteins that have been shuttled into the endosomal/lysosomal pathway. Once stable peptide/MHC class II complexes are formed, they traffic to the cell surface for presentation to helper T lymphocytes. Activated T cells specific for a particular epitope may recognize and destroy any virus-infected cell that expresses an appropriate peptide/MHC complex, but the initial activation of T lymphocytes requires an encounter with professional APCs. Professional APCs, the most prominent of which is the dendritic cell, can capture, process, and present antigens as well as provide the

necessary co-stimulation needed to activate naïve T cells. Entry of virus proteins into the MHC class I antigen presentation pathway might occur through either direct infection of APCs or by "cross-presentation" in which virus-infected cells or cellular debris are phagocytosed, and the antigens are shuttled into the MHC class I processing pathway. Recently, $CD4^+$ T cells have been shown to prime APCs via signalling through the CD40/CD40 ligand receptor pair. These activated APCs are then capable of delivering co-stimulatory signals via B7-CD28 or similar engagements for the activation of naïve $CD8^+$ T cells. Studies addressing the requirement for $CD4^+$ T-cell help for successful priming of virus-specific $CD8^+$ T-cell responses have suggested that some virus-specific responses are independent of $CD4^+$ T-cell help, likely due to alternate mechanisms for activation of APCs.[103,104]

Cellular Immune Response During Viral Lytic Cycle

It is now clear that both adaptive and innate immunity are involved in defense against viral infections. However, the participation of various arms of the immune response depends on the nature of virus-host interaction. Virus neutralizing antibodies play a definitive role in interrupting viral spread, whereas T-lymphocyte-mediated mechanisms are involved in the elimination of virus-infected target cells. The main player in this arm of effector mechanism is the $CD8^+$ T lymphocytes (CTL), which lyse virus-infected cells by recognizing an viral epitope presented by the MHC class I antigens. The effectiveness of this system depends on the nature of the epitopes that are processed from viral gene products, the presence of anchor residues that enables the MHC class I antigen to selectively bind the peptide, and transport to the cell surface for recognition by CTL. A major consideration is the presence of T-cell precursors in the repertoire. In a typical immune response to viral infection, natural killer cells that recognize virus-infected cells are recruited followed by a burst of CTL after about 4-7 days. As the virus load declines, the levels of CTL fall to a baseline that than persists as memory T cell and can be recalled upon re-exposure to the viral gene products. The CTL effector mechanism is obligatory for the elimination of virus-infected cells, especially for those viruses that cause persistent infections.[105] The CD4-mediated T-cell responses are involved in helping B cells produce antibodies and produce cytokines involved in the proliferation of $CD8^+$ T cells and activation of APC. There have been only a handful of attempts made to study the involvement of T-cell-mediated immunity

during infection of natural hosts by the three viruses, JCV, BKV, and SV40. In one report, Shah's group studied $CD4^+$ T-cell responses to BKV in seropositive individuals by the lymphocyte proliferation assay. The results showed that all of 15 seropositive individuals were positive for BKV antigens as measured by $CD4^+$ T-cell responses. Seronegative subjects were nonreactive. Unfortunately, this interesting lead has not been followed up. There may be several reasons for this. First, for human polyomaviruses there are no available, reliable animal models in which virus infection leads to its replication in vivo. However, cellular immune response to SV40 could studies in its natural host, the rhesus monkey, which is being utilized for the study of SIV. Second, the tools needed to explore this problem such as purified viruses and their components are not yet available. Third, most of the attention has been focused on T antigens encoded by these viruses as inducers of immunity against virus-induced tumors. In that arena considerable progress has been made. Tumors induced in mice by Tag expressed as a transgene have served as valuable models toward understanding a T-cell response during tumorigenesis. Another consideration that may complicate the interpretation of data regarding T-cell-mediated immunity during infection of natural hosts is the cross reactivity between both the nonvirion and virion proteins encoded by these three viruses. Despite these difficulties, the recent report by Koralnik ed al. of JCV-specific CTL in PML patients suggests a promising new approach to T-cell-mediated antiviral immunity in humans. There is a report that has addressed the role of $CD8^+$ T cells in interrupting the SV40 infectious cycle in vitro. The investigators utilized murine CTL clones specific for H2-K^b and H2-D^b restrictrd SV40 T antigen epitopes as probes for abrogating the SV40 infectious cycle in permissive monkey cells. To utilize this strategy, a continuous line of monkey kidney cells, TC-7, was tranfected with murine H2-K^b and H2-D^b class I antigens, thus allowing the presentation of T-antigen epitopes to SV40 T-antigen-specific CTL clones generated in B6 mice. The results showed that the interaction of T-antigen-specific CTL clones with SV40-infected TC7/H-2D^b or TC7/H-2K^b cells for 5 hours reduced the SV40 yield by 70-90% as measured by the infectious center assay. The results of this study lead to two important conclusions: (1) The target for CTL is a nonvirion protein, T antigen, which is synthesized before viral DNA replication; and (2) the CTL-mediated events may also take place in the natural host undergoing SV40 infection. It is also tempting to speculate

that a loss of this kindof CTL-mediated surveillance may be directly involved in the reactivation of BKV, JCV, and SV40 from latency.[106,107]

Cellular Immune Response During Viral Carcinogenesis

It is well established that SV40, BKV and JCV induce tumors in newborn hamsters and in mice when Tag is expressed as a transgene. In the latter case, Tag can induce tumors in a variety of tissues depending on the promoter used to express Tag. In addition, cells transformed by SV40 Tag are almost always transplantable in nude mice that lack T cells. In this section, we focus on the role of the immune response in carcinogenesis by SV40, as more information has been gathered with this system. However, information about BKV and JCV is included whenever relevant. Tumor induction by SV40 in hamsters was found to be age dependent. This age-related resistance to tumor induction is immunologically mediated as adult hamsters that have been immunocomprised by X-iradiation before SV40 inoculation experiments, Diamadopoulos has demonstrated that the age-related resistance could be by-passed if the hamsters were inoculated with SV40 intravenously. A wide range of tumors was observed, from leukemias to lympho-sarcomas, in these animals. Hamsters inoculated with SV40 in the cheek pouch, which is considered an immunologically privileged site, also developed tumors.[108,109] The SV40 tumors induced in hamsters have been shown generally to be transplantable in syngeneic hamsters. The first evidence of the specific antigenicity of these SV40-induced tumors was provided by a number of investigators, who demonstrated that prior immunization of hamsters with either SV40 or SV40-transformed cells lead to the development of immunologic resistance against a tumor challenge. This immunity, demonstrated by the transplantation rejection test, was specific for SV40 and mediated by T lymphocytes as treatment of SV40-immunized hamster with anti-T-lymphocyte serum abrogated the immunity against tumor challenge. Similar approaches later on were utilized to demonstrate the antigenicity of BKV- and JCV-transformed cells. Although studies carried out in inbred hamsters provided significant insights into the induction of the cellular immune response against virus specified antigenicity on SV40 transformed cells, detailed analysis of the cellular immune response and the nature of antigen could not be undertaken due to the limited knowledge about hamster immunology. In inbred mice, the inoculation of SV40 in newborns did not result in the induction of tumors in a number of mouse strains. Only in

Balb/C mice, which have been classified as low responders, did a small percentage of newborn mice that received SV40, developed tumors. In contrast, mice expressing SV40, BKV, or JCV Tag as a transgene developed progressively growing tumors in the tissue targeted for Tag expression. In transgenic mice, T-cell response is compromised by either central or peripheral tolerance. Mouse cells transformed by SV40 as a rule are not transplantable in the immunocompetent adult host as is observed in hamsters. Studies have shown that mouse cells transformed by SV40 must be cultured in vitro for prolonged time periods before becoming transplantable in syngeneic mice, and even then immunosuppression of the host is required. For example, Balb/C cells transformed by SV40 required over 50 generations of in vitro passage followed by passage in immunosuppressed hosts before inducing progressively growing tumors in adult Balb/C mice. These tumor cells maintained the expression of Tag and the MHC class I antigens. In the case of C57BL/6 mice, which have been classified as higt responders for the induction of SV40 Tag specific CTL, SV40-transformed cells only become transplantable in syngeneic mice at the expense of H-2^b class I antigen and only after prolonged in vitro culture and passage in newborn mice. These observations indicated that SV40, as well as BKV and JCV, transformed cells contain strong antigenicity resulting in the induction of a highly effective cellular immune response in the host.[110,111]

1.6.1 Tag as a target for T-cell-mediated cellular response

SV40, JCV, and BKV Tag are largely homologous in structure and function and are the first virus-encoded non-structural protein synthesized by the infected cells during permissive and nonpermissive infection. The Tag is a highly immunogenic protein that induces both a humoral and cellular immune response in the host. Because Tag and tag, also encoded by the SV40 early region, are the only proteins synthesized in the transformed or tumor cells, it is logical to assume that either of these two proteins plays a dominant role in inducing the immune response and thus are the targets for cellular immune response mediated by T lymphocytes. A large amount of data published during the last 25 years have definitively shown that Tag is the target for immune response and participates in inducing tumor rejection in the experimental host preimmunized with SV40.[112] BKV and JCV are expected to behave similarly. The

earlier evidence for the role of Tag can be summarized as follows. (1) Cells from different species transformed by SV40 and expressing Tag share the ability to immunize against the transplantation of tumors induced by SV40 in either hamsters or mice. Interestingly, a number of studies carried out with BKV, JCV, and SV40 transformed cells observed cross reactivity between the CTL epitopes encoded by these viruses. (2) Adenovirus-SV40 hybrid viruses, which express either full-length Tag or a fragment of Tag, were able to confer immunity to hamsters and mice against tumor transplantation. This finding suggested that the Tag is responsible for inducing the rejection response. In addition, Tag synthesized during the permissive cycle also induced tumor immunity. (3) Partially purified Tag induced an effective response in syngeneic mice against SV40 tumors in vivo, followed by the demonstration that the Tag purified to homogeneity not only induced SV40-specific tumor immunity in Balb/C mice, but in high responder C57BL/6 mice induced a cytotoxic T-cell response specific for SV40 Tag. This later observation identified Tag as responsible for inducing both a T-cell response and tumor immunity. Further evidence that Tag directly participates in priming the cellular immune response came from the finding by Flyer et al. that a loss of SV40 Tag expression in polyomavirus-induced tumor cells expressing SV40 Tag when subjected to immunologic pressure in vivo led to the loss of recognition by the SV40 Tag specific CTL as a result of the loss of Tag itself.[113] The generation of CTL specific for SV40-transformed cells has been demonstrated in a number of studies in which high responder mice of C57BL/6 origin (H-2^b) upon immunization with either syngeneic or allogeneic transformed cells develop CTL specific for SV40 Tag in an MHC class I-restricted manner. These observations combined with the direct demonstration of Tag as the target for CTL have led to the understanding that CTL are the major player in inducing immune resistance against tumor development. The understanding of MHC restriction, the development of CTL clones specific for Tag, and the demonstration that short synthetic peptides of 8-10 amino acids are presented by the MHC class I molecules to the CTL led to the identification of CTL epitopes in SV40 Tag.[114,115] Initial isolation of two distinct CTL clones from H-2^b mice immunized with syngeneic SV40 Tag transformed cells that differed in their specificities for the recognition of SV40 and BKV Tag epitopes and their close proximity in the amino terminal half of Tag provided the first evidence that the Tag posseses multiple epitopes and that CTL specificity may

be dictated by the amino acid sequence. Using a large collection of CTL clones generated in B6 mice immunized with SV40-transformed B6 cells, four distinct epitopes were identified that were specific for SV40 Tag. By using the Tag deletion mutants truncated at either carboxy or amino terminal ends or carrying internal deletions and using synthetic peptides, the four CTL epitopes were mapped to residues 206-215 (epitope I), recognized by CTL clones Y-1 and K11; residues 223-231 (epitope II/III), recognized by CTL clones Y-2 and Y-3; residues 404-411 (epitope IV), recognized by CTL clone Y-4; and residues 489-497 (epitope V), recognized by CTL clones Y-5 and H-1. The epitopes I, II/III, and V are H-Db restricted, whereas epitope IV is H-K^b restricted. The multiplicity of CTL epitopes on Tag raises the question of the immunodominant and immunorecessive nature of these CTL epitopes within Tag and their relative roles in inducing a class I-restricted CTL response. An immunologic hierarchy has been demonstrated among these four Tag epitopes. Immunization of B6 mice with full-length Tag expressed either in transformed cells or cloned in a vaccinia virus vector leads to the induction of CTL specific for epitopes I, II/III, and IV but not V as judged by the frequency analysis for pre-CTL specific for each of the epitopes. However, B6 mice immunized with Tag from which epitopes I, II/III, and IV have been inactivated by site-directed mutagenesis or by deletion did induce CTL specific for epitope V. This example of immunodomination has important implications for the use of immunotherapeutic approaches. Interestingly, CTL epitopes preceeded by a signal sequence that allows direct access of the epitope peptide into the ER and expressed in a recombinant vaccinia virus induced CTL to epitope V extremely efficiently.[116,117]

1.6.2 Cellular immune response in SV40 Tag transgenic mice

Expression of the Tags from SV40, JCV, and BKV as a transgene in mice can induce tumors at distinct anatomic sites depending on the promoter used to drive expression of the transgene. These mice provide powerful tools to examine the effect of endogenous Tag expression and tumor progression on the immune response. Expression of SV40 Tag in vivo can have varied effects on $CD8^+$ T-cell immunity, ranging from the spontaneous perturbation of autoimmune disease to the induction of profound tolerance, depending on the site and timing of Tag expression. Because detailed studies on the immune responsiveness of BKV and JCV Tag transgenic mice remain to be performed,

this section focuses on results obtained with SV40 Tag transgenic mice. Expression of Tag in the thymus during T-cell development most often results in the loss of Tag epitope-specific T cells by clonal deletion. Potentially autoreactive T cells do survive negative selection in some Tag transgenic mouse models where Tag expression occurs after T-cell development. Such models have been used to study the effect of Tag induced peripheral tolerance for both $CD4^+$ and $CD8^+$ Tag specific T lymphocytes. Flavell and co-workers described a system in which the fate of H2-K^k-restricted SV40 Tag specific T-cell receptor (TCR) transgenic T cells was determined in line 177-5 ($H2^k$) mice expressing SV40 Tag from the rat elastase I promoter. Although expression of the transgene was detected in the thymus as well as the pancreas, TCR transgenic $CD8^+$ T cells were positively selected and entered the periphery where they were involved in the establishment of autoreactivity in the pancreas at an early age. These Tag specific $CD8^+$ T cells, however, became anergic and disappeared from the periphery by 5 months of age, after which panvreatic tumors became apparent. This onest of anergy was linked to events that occurred in the thymus before exit of the T cells into the periphery, resulting in a gradual loss of T-cell responsiveness to Tag. A different scenario was observed for RT3 mice, which express Tag from the insulin promoter. In RT3 mice crossed with H2-K^k-restricted TCR transgenic mice, the $CD8^+$ T cells develop normally and show no apparent signs of tolerance in the periphery even after 5 months. This might be attributed to the delayed expression of Tag in RT3 mice, which is not detected until 10-12 weeks of age. These bouble transgenic mice show signs of autoimmunity in the pancreas similar to that of young line 177-5 mice, suggesting that the mechanisms responsible for the maintenance of peripheral tolerance cannot be maintained in the presence of a large population of potentially autoreactive T cells. In contrast, TCR transgenic $CD8^+$ T lymphocytes ignored expression of the H2-K^k-restricted CTL epitope in the islet cells of mice expressing a nontransforming Tag fragment from the insulin promoter.[118,119] Lack of spontaneous autoimmunity in this model was attributed, in part, to the absence of signals that could occur if transformed cells were present. Co-expression of the B7.1 co-stimulatory molecule on the pancreatic islet cells with the Tag fragment resulted in destruction on the pancreatic islet by activated TCR transgenic T cells, indicating an inherent lack of co-stimulation for the activation of potentially autoreactive T cells in this model. These results indicate

that SV40 Tag expression in mice can result in autoimmunity, immunologic ignorance, or antigen-induced tolerance. The role of Tag specific immunity in the control of spontaneous tumor progression in Tag transgenic mice was addressed using the RIP1-Tag4 ($H2^b$) line of mice, which express full-length Tag from the insulin promoter. Tag is detected in the pancreas at 10-12 weeks of age in RIP1-Tag4 mice. This expression results in the progressive growth of insulinomas that become life threatening by 8-9 months of age. RIP1-Tag4 mice develop functional Tag specific $CD8^+$ T cells that can be activated by specific immunization with SV40 to control the progressive growth of tumors if the mice are immunized before 9 weeks of age. Immunization after 9 weeks of age failed to induce a significant delay in tumor progression. Whether this failure is due to the gradual onset of tolerance with increasing age similar to that which occurs in line 177-5 mice was not directly determined. The insulinomas in RIP1-Tag4 mice, however, were shown to down-regulate MHC class I expression, which is a common mechanism of escape from CTL-mediated immunity for some tumors. The ability of individual CTL epitopes to induce the control of spontaneous tumor progression was investigated in SV11 mice that express Tag as a transgenic from the SV40 enhancer/promoter and develop choroids plexus papillomas. SV11 ($H2^b$) mice are tolerant to the immuno-dominant Tag epitopes I, II/III, and IV due to expression of Tag in the thymus during T-cell ontogeny. CTL specific for the immunodominant H2-K^b-restricted epitope IV were established in SV11 mice following adoptive transfer of naive C57BL/6 splenocytes following a nonlethal dose of γ-irradiation resulted in priming of epitope IV-specific CTL against the endogenous Tag and a highly significant increase in the life span of SV11 mice due to inhibition of tumor progression. In similar studies by others adoptive transfer of Tag immune lymphocytes or a CTL line specific for Tag epitope II/III has been shown to moderate the progressive growth of Tag induced prostate and liver tumors, repectively, in Tag transgenic mice. The fate of endogenous CTL specific for individual $H2^b$ epitopes was examined in the 501 lineage of mice that express Tag from the late liver α-amylase promoter and is first detected in the salivary glands at 3 months of age. After 8 months of age 501 mice develop Tag expressing osteosarcomas that can metastasize to the liver and lungs. Spontaneous autoimmunity was not detected in 501 mice, in contrast to some of the Tag transgenic mouse models discussed above. Immunization of 501 mice with rVVs expressing individual Tag

epitopes reveled a sequential loss of CTL responses against the Tag epitopes. Loss of epitope I-specific CTL occurred by 6 months of age, corresponding with increased levels of Tag expression in the salivary glands. Importantly, loss of epitope IV-specific CTL responses correlated with the appearance of Tag expressing osteosarcomas. The use of MHC class I/epitope IV tetramers revealed that there was a progressive decrease in the number of epitope IV-specific $CD8^+$ T cells that could be recruited by immunization, culminating in complete loss of responsiveness with the appearance of tumors. These results indicate that autoreactive and potentially tumor-reactive T lymphocytes are tolerized over time due to the endogenous expression of Tag. This tolerance likely occurs through cross-presentation of the antigen, which leads to either deletion or the induction of T-cell anergy as has been described for other transgenic systems. Fewer studies have addressed the role of Tag specific $CD4^+$ T cells in the control of Tag induced tumors in transgenic mice. Two studies directly addressed the role of Tag epitope-specific $CD4^+$ T cells in the control of insulinomas using the RIP1-Tag5 (RT5) line of mice, which express full-length Tag from the insulin promoter at 10-12 weeks of age in the pancreas. RT5 mice succumb to tumors at approximately 4-5 months of age. RT5 mice were crossed with TagTCR2 mice, which express a transgenic TCR specific for an $H\text{-}2^k$-presented $CD4^+$ T-cell epitope in order to increase the frequency of antigen-specific $CD4^+$ T cells. While there was an increase in the degree of infiltration of Tag expressing islet cells in these double transgenic mice, no effect in tumor progression was observed. These endogenous Tag specific $CD4^+$ T cells could be activated in vivo if B7.1 also was expressed on pancreatic islet cells, resulting in insulitis and diabetes before tumor formation. In contrast, adoptive transfer of activated lymphocytes from normal or Tag TCR2 transgenic mice into tumor-bearing RT5 mice resulted in a significant infiltration of early-stage tumors if the recipient mice were first irradiated. The authors suggest that irradiation might contribute to increased permeability of the tumors. These studies support the idea that presentation of Tag epitopes in the absence of proper co-stimulation leads to tolerance instead of T-cell activation. The effect of the endogenous expression of BKV or JCV Tag on Tag specific immunity remains to be determined. Several lines of BKV and JCV transgenic mice have been developed and await investigation of immune function and the role of immunity in control of Tag induced tumors. Mice expressing the BKV Tag from the

viral promoter developed Tag expressing primary renal and hepatocellular tumors as well as thymic hyperplasia and thymomas. Mice expressing JCV Tag develop neuroectodermal tumors and metastatic adrenal neuroblastomas. The status of the immune response awaits elucidation in these models.[120-123]

1.6.3 Cross reactivity among SV40 Tag with BKV and JCV Tag CTL epitopes

Understanding CTL epitope specificity of SV40, BKV, and JCV Tag is important as the Tag of these viruses show considerable amino acid homology, and all three viruses have now been isolated from humans. The epitope specificity also provides a measure of selective cellular immune response to each of these viruses. An understanding of this immune response will allow the elucidation of the level of immunosurveillance not only against virus infections but also against neoplastic development. The selection of CTL epitopes is dictated by the rules of determinant selection, which include the presence of anchor residues specific for each of the MHC class I haplotypes. In the case of SV40 Tag, epitopes have been more thoroughly defined for the H-2^b haplotype. Thus, for a peptide to be presented by the H-$2D^b$ molecule, it needs an asparagines residue at position five and a hydrophobic residue at position 9 at the carboxy-terminal end, whereas for phobic residue at position eight at the carboxy terminus. In addition to having the proper MHC class I binding motif, there are other requirements that affect the generation of effector T cells. Our approach to discriminating among Tag encoded by SV40, JCV, and BKV is based on the fine specificity of SV40 Tag CTL clones. Only those epitopes in BKV and JCV Tags that show identity in critical amino acids with the corresponding SV40 Tag will be recognized by the SV40 Tag specific CTL clones. We therefore compared the sequences of SV40 Tags epitopes I, II/III, V, and IV with the corresponding sequences in Tags encoded by JCV and BKV and tested the reactivity of SV40 T-antigen-specific CTL clones directed epitopes I, II/III, V, and IV with cells transformed by SV40, JCV, or BKV Tags. This panel of CTL clones allowed us to distinguish between the Tags expressed by SV40, JCV, and BKV. In the same study, we determined that three viruses isolated from humans are indeed authentic SV40. One important step was to transform primary mouse cells of C57BL/6 origin (H-2^b) with the human isolates. The use of C57BL/6 cells was necessary because the CTL clones that recognize SV40 Tag epitopes are H-$2D^b$ and

H-2K^b restricted, and for this reason the CTL epitopes processed from Tag must be presented by mouse (H-2^b) MHC class I molecules. It is possible, nonetheless, to test cells derived from human tumors suspected of expressing a papovavirus Tag for the presence of CTL epitopes detected by the CTL clones by expressing H-2K^b and/or H-2D^b genes into the tumor cells. Alternatively, the H-2K^b and/or H-2D^b gene products could be introduced by infecting the tumor cells with vaccinia virus vectors that express these genes. Thus, the approach we have used to document the identity of the papovavirus Tag in human tumors need not rely on isolating infectious virus or amplifying Tag coding sequences from the tumors.[124]

1.6.4 Antibody responses during virus infection in the permissive host

The natural host for SV40 is rhesus monkey, and the virus appears to be latent in the kidneys. The antibody response to SV40 in these monkeys and virus transmission in the monkey colonies have been nicely summarized by Shah and Nathanson (1976). Based on the antibody profiles, the rhesus and a few other macaques are susceptible to SV40 infection. As adults, a large proportion of rhesus monkeys become seropositive for SV40. Seronegative rhesus monkeys can be infected with SV40 via a variety of routes and demonstrate viremia.[125] An earlier study showed that African green monkey inoculated with autologous cells infected with SV40 developed antibodies to the virus as well as Tag. No sign of neoplasia was noticed at the site of inoculation in these monkeys, again showing that the development of antibodies to Tag is not a sign of neoplasia. Infection of human by BKV and JCV is widespread, and most of the young population develop antibodies to these two viruses.[126]

1.6.5 Antibody responses in semipermissive host

In an attempt to understand antiviral immune response in a permissive host, rabbits, which are considered a semipermissive host, were inoculated intravenously with either a high dose (1×10^{12}) or a low dose (1×10^{9}) of purified SV40 and were studied for the development of antibodies to Tag, virion antigen, and virus neutralizing antibodies for various periods of time. Rabbits inoculated with a high dose of SV40 synthesized high levels of virus neutralizing antibodies as well as antibodies to Tag. This pattern of antibody response lasted for up to 200 days of the observation period. Rabbits

inoculated with a low dose of virus synthesized antibodies to the virus only after a re-challenge with a high dose of virus. These results suggested that SV40 establishes a persistent infection and continues to produce virus and Tag. Any virus released from the cells is neutralized by antiviral antibodies. These results further suggested at that time that the presence of antibodies to Tag does not necessarily indicate the presence of Tag induced tumor. It merely indicates that a virus infection has occurred that may or may not lead to transformation of cells. With a number of reports documenting the detection of SV40 DNA sequences in a variety of human tumors and in some cases the synthesis of Tag, it is quite likely that a select population of humans may become exposed to SV40. However, SV40 administered to humans as a contaminant of polio and RSV vaccines is capable of inducing SV40 neutralizing antibodies that persist in some cases for prolonged periods, suggesting that virus may have established a persistent infection. Viral neutralizing antibodies were induced by either the subcutaneous or intranasal route but not by the oral route. Whether SV40 induces a clinical disease similar to BKV or JCV remains to be established.[127,128]

1.6.6 Antibody responses to Tag during tumorigenesis in nonpermissive hosts

SV40 Tag was identified in SV40-transformed or tumor cells by the complement fixation test using sera from hamsters bearing SV40 tumors. The nuclear location of Tag was documented by the immunofluorescence test. The almost universl development of high titer antibodies to Tag in hosts bearing tumors induced by virus-free transformed or tumor cells demonstrated that the Tag is a highly antigenic protein and is capable of inducing the antibody response during tumorigenesis either by the virus or virus-induced tumor or transformed cells. Hamsters and mice bearing tumors expressing SV40 Tag respond by making antibodies not only to Tag, mostly clustered in the amino- and carboxy-terminal ends of Tag, are recognized by antibodies from the tumor-bearing host. The multiplicity of epitopes in Tag is supported by the isolation of a large group of monoclonal antibodies directed to different specificities by a large group of investigators.[129] The immunogenicities of BKV and JCV Tags are essentially very similar to that of SV40 Tag. There is extensive cross reactivity between epitopes of BKV, JCV, and SV40 Tag epitopes. Only some of the monoclonal antibodies made against SV40 Tag are specific for SV40 Tag. JCV Tag specific monoclonal antibodies

were isolated by immunizing SV11 Tag transgenic mice that are tolerant to epitopes on SV40 Tag with JCV-transformed mouse cells followed by selection of JCV Tag specific antibodies. The JCV Tag specific epitopes were localized in the amino terminus of Tag. Isolation of other JCV-specific antibodies also has been reported.[130]

1.6.7 Immunologic approaches for associating SV40 with neoplasia in humans

SV40 is being associated with the development of a number of human neoplasias (mesothelioma, osteogenic sarcomas, and others) based on the presence of Tag sequences amplified by PCR. In only a few cases the expression of Tag protein has been demonstrated. However, the role of SV40 as an etiologic agent of human neoplasia remains to be firmly established. A large number of studies using experimental models have shown that adult rodents exposed to SV40 are resistant to tumor development largely due to a strong specific immunosurveillance mediated by the cell-mediated immune responses against any emerging potentially transformed cells in vivo. However, differences in the mode of exposure might influence the outcome. In certain cases where the virus is administered by alternative routes, adult hamsters develop a variety of tumors. Studies in Tag transgenic systems have shown that T-cell responses specific for Tag are silenced by tolerance before tumor progression. Applying the immunologic findings from the experimental SV40 tumor system to the involvement of SV40 Tag in tumors in human will provide convincing supporting data either for or against SV40 as an etiologic agent. There are three host-SV40 interactions that may lead to the development of immune responses to either viral or Tags. The first is the transient infection of SV40 in humans without virus persistence or establishment of transient foci of persistently infected or transformed cells. This abortive infection may not provide a sufficient antigenic load to trigger an immune response either to virus proteins or to Tag. It is possible that multiple abortive exposures by SV40 in the same individual may lead to the induction of virus neutralizing antibodies that are sufficient to control any future infection by SV40. In the case, the viral antibody titers are likely to remain low or undetectable. However, the studies reported in the literature have detected significant titers of virus neutralizing antibodies in children.[131,132] An intriguing possibility is that the virus may target dendritic cells, the professional APCs. These cells, after processing the antigen, viral or Tag, will trigger an efficient immune response by sensitizing both

CD8 and CD4 T cells. Viral or Tag produced from cells that are not APC must undergo cross-priming by the APC, a process that may compromise the efficiency antigen presentation. Because persistence of memory T cells does not require a continuous antigenic stimulus, their presence in the circulation of these T cells can nevertheless be detected and quantitated by the sensitive, highly specific in vitro techniques currently available. If the virus targets a tissue such as kidney, there is the likelihood that a massive replication of the virus can occur, such as is seen in infections in human by BKV, until the virus undergoes latency. In that case, a strong immune response will result in the synthesis of antiviral antibodies and a T-cell response to Tag as well. Tag synthesis in the kidneys has been demonstrated in patients under immunosuppression. The problem of defining humans at risk of SV40 infection is that virus has not been linked to a clinical syndrome, whereas BKV and JCV have been implicated directly and routinely with nephropathy and PML, respectively. The second type of virus-host interaction is the establishment of persistent infection in which cells will continue to synthesize Tag as well as viral proteins with some production of virus that could infect other susceptible cells. In this case, a strong antibody immune response to both the virus and Tag will occur. In addition, a vigorous cellular response will occur. These responses will be maintained at high levels due to continuous antigenic stimulation. Neutralizing antibodies will eventually terminate the persistent state, and the virus may undergo latency that may lead to down-regulation of immune responses. Reactivation of the virus under immunosuppression may restart the cycle. In this context, it is interesting to note that transplant patients have been shown to develop high neutralizing antibody titers to SV40. The third type of SV40-host interaction may be establishment of fully transformed cells that are capable of forming a foci that may develop into a progressively growing tumor accompanied by other cellular changes. In this case, the Tag in the transformed cells will induce a vigorous antibody response. Experimental evidence suggests that the antibody titers correlate with the size of the tumor and, in later stages, the T-antibody titers may drop due to the formation of immune complex. In addition, the antibody responses may spread to epitopes other than the dominant epitopes in the carboxy and amino termini of Tag. The development of antibody to Tags in this type of interaction will depend on the synthesis of adequate levels of the proteins by the transformed or tumor cells. Furthermore, antibodies to small tag specific epitopes

may also develop in the host. For the detection of Tag in potential tumor cells, it is essential to use well-defined monoclonal antibodies directed to specific epitopes. In addition, it is desirable to use monoclonal antibodies that recognize both ends of Tag. Efforts should be made to utilize monoclonal antibodies that are specific for SV40 Tag. A large number of monoclonal antibodies that are made against SV40 Tag have shown to cross react with BKV and JCV Tags. It would also be desirable to use antibodies that work well in all types of assays. For the detection of antibodies to Tag in human sera, care should be taken to include adequate controls to eliminate non-specific reactions. For the detection of antiviral antibodies in a patient's serum, strict specificity controls are needed to differentiate reactivities between SV40, JCV, and BKV. It would also be desirable for all investigators to utilize a standard strain of SV40 and to utilize purified viruses. The last point is important for the measurement of antiviral antibodies by ELISA. Tracking cellular immune responses in human suspected of being infected with SV40 or patients with cancer in whom SV40 in suspected as the etiologic agent would provide substantial supporting and convincing evidence for a role of SV40 in human neoplasia. The presence of $CD8^+$ T cells specific for epitopes for Tag un human will suggest that infection by SV40 has occurred and that the expression of Tag would induce a cellular response. The magnitude of CD8 T cells responding to Tag might indicate a continued antigenic stimulation. To explore the CD8 T-cell responses in human, it is essential to seek an experimental approach that would identify HLA-restricted Tag epitopes. Our laboratory utilized HLA-A2.1 transgenic mice that have been shown to respond to HLA-A2.1-restricted T-cell epitopes in influenza virus and human p53.[133,134] HLA-A2.1 transgenic mice were immunized with B6 cells transformed by Tag from which the known $H\text{-}2^b$-restricted epitopes were inactivated by mutagenizing the anchor residues. $CD8^+$ T cells from these mice were shown to be Tag specific but only in association with the HLA-2.1 class I antigen. Thus, any cell expressing HLA-A2.1 and Tag would be lysed by the CTL clone that in specific for Tag in association with HLA-A.2.1. The HLA-A.2.1-restricted epitopes was mapped to Tag residues 281-289. This epitope represents a potential specific CTL recognition epitope for human. It was interesting to note that the 281-289 Tag epitope did not cross react with either BKV or JCV. By synthesizing the HLA-A.2.1/T281 tetramers, it was possible to quantitate the number of $CD8^+$ T cells in HLA-A.2.1 transgenic mice

immunized with Tag. This powerful approach will allow investigators to track SV40 Tag specific T-cell responses at least in HLA-A.2.1-positive individuals. This approach will also allow the identification of HLA-A.2.1-restricted epitopes specific for BKV and JCV Tags. This may be an additional way to distinguish between SV40, BKV, and JCV.

1.7 Epidemiology of SV40 infection in humans

SV40 natural infection in human is considered a rare event, restricted to people living in contact with monkeys, the natural hosts of the virus, such as inhabitants of Indian villages located close to the jungle, and workers attending to monkeys in zoos and animal facilities. A source of human exposure to SV40 occurred between 1955 and 1963, when inactivated and live anti-polio vaccines, prepared from polioviruses grown in naturally SV40-infected simian cell cultures, were administered to hundreds of millions of people in the United States, Canada, Europe, Asia and Africa. Soon it was shown that children vaccinated with contaminated oral polio vaccines (OPV) shed infectious SV40 in stools for at least 5 weeks after vaccination. However, some children, who received the same OPV, did not develop neutralizing antibodies even though they may have received large doses of live SV40, compared with the potentially inactivated SV40 in inactivated polio vaccine (IPV). Further, SV40 human contamination occurred in experimental infection with live respiratory syncytial virus to adult volunteers and a neutralizing antibody response in about two thirds of the volunteers was shown. Inactivated vaccines against adenoviruses and hepatitis A virus also exposed humans to SV40, although the amount of infectious SV40 was almost certainly lower then that administered with OPV or live respiratory syncytial virus.[135,136]

Early serologic studies reported the presence of SV40 neutralizing antibodies, at different titles, in the population that received IPV. Immune response appeared to correlate with the amount of SV40 present in the vaccine; 30% to 50% of individuals reached a significant antibody response against formalin-inactivated SV40 after three doses of the vaccine. Antibody titles persisted for a period of up to 3 years post-inoculation. Additional serologic studies reported a SV40 seropositivity in individuals with no history of immunization with contaminated IPV or other possible route of SV40 infection. Shah et al. detected antibodies to SV40 in children born after 1964, when IPV was free of SV40, as well as in people born before 1954. These studies suggest that

humans may become infected by SV40 independently from poliovirus vaccine exposure. However, most of these early serologic studies were carried out before the discovery of the two human polyomaviruses, BKV and JCV, which are close related to SV40 and are ubiquitous in human populations. It is possible that the early serologic evidence of SV40 antibody detection in human sera represents some degree of cross reactivity with antibodies against the highly related BK and JC viruses.[137,138]

To date, the prevalence of SV40 infections in humans is not known. Recent studies, based on PCR and serological techniques, indicate that SV40 infection occurs both in children and adults. (i) SV40 DNA sequences have been detected in normal and neoplastic tissues of people either too young (1 to 30 years) or too old (60 to 85 years) to have been vaccinated with SV40-contaminated anti-polio vaccines. This finding may also explain the lack of difference in cancer incidence between individuals vaccinated with SV40-contaminated and SV40-free anti-polio vaccines. (ii) SV40 sequences and Tag were detected in blood and sperm specimens from normal individuals and oncologic patients and in lymphoblastoid cells. These results suggest that PBMCs could be a reservoir and vehicle of SV40 spreading in the tissues of the host and among the individuals. (iii) SV40 sequences were found in urine and stoole samples, from children and adults, indicating that the haematic, sexual and orofecal routes of transmission are likely to be responsible for SV40 horizontal infection in humans. (iv) Infectious SV40 was rescued by transfection of permissive CV-1 monkey cells with the DNA of an SV40-positive human choroid plexus carcinoma, one blood and one HPV-infected normal vulvar tissue samples. Finally, specific antibodies to SV40 capsid antigens have recently been found in human sera. Unfortunately there is no comparative data on the SV40 DNA prevalence in PBMC and antibodies presence to SV40 antigens in sera from the same patients.[139]

Among recent SV40 serologic investigations, some data suggest that specific SV40 antibodies are present in human sera: (i) in a collection of human sera from Morocco, 100% of the samples had antibodies to SV40, whereas in the same study other sera from Morocco, Zaire, Sierra Leone and Poland contained SV40 antibodies in 0.4 to 5.3% of the samples, a figure which corresponds with the results of other surveys. All the sera in the 100% positive Moroccan collection were from cases of poliomyelitis in children under five years of age. These children, therefore, had probably not been vaccinated

against poliovirus. This result should not be overestimated, especially because the collection of the 100% anti-SV40-positive sera was made up of only 29 samples. Nevertheless, this observation suggests that, under particular circumstances, humans can display a great specific antibody response to SV40. Perhaps, the overt poliomyelitis syndrome has influenced the immunological reaction of affected patients to SV40. (ii) Although it is obviously difficult, due to the ubiquity of the two human polyomaviruses, to find a human serum positive for SV40 and negative for both BKV and JCV antibodies, one such serum was detected. (iii) While seroconversion to BKV and JCV is age-dependent, there is no data on the age-dependent seroconversion to SV40, suggesting that most of the SV40 antibodies present in human sera are not generated by infection with BKV or JCV. (iv) In sera from two immunosuppressed renal transplant patients, that were examined sequentially for antibodies to BKV, JCV and SV40 over a period of 82 and 51 weeks, respectively, a significant rise in SV40 antibody titers was detected, indicating that a latent SV40 infection, like BKV and JCV latent infection, can be reactivated in humans by immunosuppression. Moreover, during the post-transplant follow-up, the evolving profile of antibodies to SV40 was clearly different from that of antibodies to either BKV or JCV, suggesting a specific immunological response to SV40 in these two patients. Some other recent SV40 serological studies, using virus neutralization and ELISA test, showed antibodies to SV40 in a limited number (1.3 to 15.6%) of normal human sera, suggesting a low virus circulation in the human population. Other studies have examined seroprevalence for SV40 in cancer patients compared with controls using viral-like-particle (VLP)-based assays, which detect antibodies that are specific to the major capsid protein VP1 of SV40, BKV, and JCV. These studies showed that SV40 seroprevalence is similar between cancer patients compared with controls (ranging from 5% to 10%) and suggest that no association exists between SV40 seroprevalence and either immunization with poliovirus vaccine or cancer incidence.[140,141]

The antigenic cross-reaction of SV40 with the two human polyomaviruses BKV and JCV has been, so far, the most difficult problem in studying the real diffusion of SV40 infection in humans. Carter et al.[142], using recombinant SV40 VP1 virus-like particles (VLPs) as antigen in an ELISA test, detected antibodies to SV40 in 6,6% of human sera, whereas SV40 reactivity in the same sera disappeared after serum pre-adsorption with

BKV and JCV VLPs. Due to the different results obtained before and after pre-adsorption with BKV and JCV VLPs, this study suggests that most of the seropositivity for SV40 is actually caused by cross reactivity to BKV or JCV. The authors concluded that authentic SV40 antibodies are absent in human sera and therefore SV40 does not appear to be a prevalent human pathogen. However, some considerations can be made on the validity of SV40 studies using VLPs. Due to the sequence homology of the VP1 structural proteins (i.e., more than 80% identical) in the three polyomaviruses, BKV, JCV, and SV40, it is not surprising that the vast majority of the human antibody repertoire against the VP1 protein of the three polyomaviruses can largely overlap. This cross-reactivity, in turn, increases the probability that immunodominant epitopes are present within the family of VP1 antigenic determinants common to all three polyomaviruses. Coexistence of immunodominance and cross-reactivity has been largely documented in the literature. Thus, both qualitative and quantitative differences in the antibody response to the epitopes present only in the SV40 VP1 structural protein may be difficult to determine, particularly if an assay based on serum preadsorption with BKV and JCV VLP is used as the sole means of detection. Therefore, due to the great homology of the VP1 structural protein in the three polyomaviruses, preadsorption with BKV and JCV VLPs may have removed from human sera most of the SV40 antibodies, which cross-reacted with human polyomavirus capsid antigens. Furthermore, it is worth noting that ELISA tests can detect non-neutralizing antibodies and some cross-reactivity is not unexpected among the three polyomaviruses. In theory, VLPs should resemble native virions and retain some of their immunological features. However, polyomaviruses VLPs based assays used the VP1 capsid protein, a highly homologous structural protein among the three viruses. Altogether these considerations suggest that the validity of SV40 studies using VLPs assays is problematic.[142,143]

The site of SV40 latent infection in humans is unknown. Detection of SV40 in human kidney and urine points to the kidney as a site of virus latency, like in the natural monkey host. Clues to the mechanisms of SV40 transmission in humans may come from studies of the natural infection in monkeys. Since uninfected weaning animals do not frequently seroconvert when grouped with the infected mothers or littermates, it seems most likely that transmission of SV40 in monkeys, under conditions of natural infection, occurs after weaning from the environment rather than directly from

other animals. Interestingly, this observation would support SV40 transmission in humans from the contaminated general environment or from the home environment. Finally, SV40, as with other tumor viruses, tends to establish long-term persistent infection, as compared with the self-limited infection typical of most common viruses. Therefore, the response of the host exerts constant pressure on chronic virus infection and, in defence, viruses contain genes that have the potential to modulate such host responses. Indeed, recent data indicate that SV40 miRNAs downregulate Tag, a target of the cytotoxic T lymphocyte (CTL) response expression, promoting the CTL evasion in "in vivo" conditions. Thus, down-regulating the accumulation of unnecessary Tag, the SV40 miRNAs reduce CTL susceptibility and local cytokine release. Although this down-regulation is dispensable for viral growth in culture, it is likely to be of considerable importance "in vivo". Predicted hairpin structure for the pre-miRNA is not only found in all SV40 isolates, but also conserved in other primate polyomaviruses, including BKV and JCV.[144-146]

1.8 SV40 in human non-malignant specimens

SV40 sequences were detected in kidney and cells of urine sediments from patients with focal segmental glomerulosclerosis and SV40 was isolated by co-cultivation of cells from urine sediments of such patients with CV-1 monkey cells. SV40 DNA was localized to renal tubular epithelial cell nuclei in renal biopsies of patients with focal segmental glomerulosclerosis as determined by in situ hybridization. Several strains of SV40 were rescued in this study, including strain 776 and other strains bearing mutations in the early and late regions. One study reported that SV40 and BKV sequences have been co-detected in the kidneys of patients with post-transplantation interstitial nephritis thus suggesting that SV40 may cooperate in the etiopathogenesis of this chronic disease. Moreover, the presence of SV40 in kidney tissue and urine points to the kidney as a site of virus latency, like in the natural monkey host. Other studies showed that SV40 DNA sequences from the viral regulatory region were detected and identified in the allografts of immunocompromised pediatric renal transplant recipients and in the native kidneys of a young adult lung transplant patient with polyomavirus nephropathy. Different studies have detected SV40 DNA sequences in PBMCs from various patient populations. These results demonstrate the nephrotropic and lymphotro-

pic properties of SV40 and indicate that the kidney can serve as a reservoir for the virus in humans. It appears that patients with acquired and/or iatrogenic immunosuppression are a population at risk for SV40. However, the frequency, natural history, and morbiddity of the virus in this increasing patient population are unclear.[147]

1.9 Association of SV40 with human tumor specimens

SV40 sequences have been found, mainly by PCR methods, in different human cancers including mesothelioma, osteosarcoma, and non-Hodgkin's lymphoma and some different lymphoproliferative disorders, a variety of childhood brain tumors such as ependymoma and choroid plexus tumors, as well as thyroid, pituitary and parotid gland tumors. These human tumors correspond to the neoplasms that are induced by SV40 experimental inoculation in rodents or by generation of transgenic mice with the SV40 early region gene directed by its own early promoter-enhancer. SV40 sequences were detected in most cases by PCR. However, in two independent studies SV40 sequences were detected, by Southern blot hybridization, integrated in human osteosarcomas and thyroid tumors. In addition, infectious SV40 was isolated from a choroid plexus carcinoma as well as from one blood sample and one HPV-infected vulvar tissue sample. However, other studies examining polyomaviruses in brain cancers using primers capable of detecting the three polyomaviruses have not reported co-infection. Different types of lymphomas and other lymphoproliferative disorders, from immunosuppressed/ HIV+ patients, tested positive for SV40 sequences and Tag expression. However, the SV40 prevalence in lymphomas from immunosuppressed/HIV+ and HIV- oncologic patients did not differ substantially. The semi-permissiveness of human cells to SV40 infections may explain the restricted multiplication of the virus even in the immunosuppression condition of the host.[148,149] Some of the above mentioned studies also demonstrated the messenger RNA of SV40 Tag by RT-PCR and/or the viral oncoprotein presence by immunohisto-chemistry in tumor tissues. On the contrary, other studies failed to demonstrate both SV40 sequences and Tag protein using similar technical approaches in the same tumor kind. It has been suggested that different techniques employed in DNA extraction and purification from human specimens may account for the SV40-negative data. Indeed, some popular commercial kits do not allow the recovery of the small SV40 DNA (5.2 kb) when present in a low amount and in the

episomal state. In addition, it has been reported that SV40 DNA sequences could be amplified by PCR with certain sets of primers but not with others. These discrepancies have led to questions regarding the sensitivity and specificity of PCR-based detection of SV40 as well as the possibility of false-positive results caused by laboratory contamination. To settle this dispute, two multi-institutional studies were performed to examine the presence of SV40 in human malignant mesotheliomas. Unfortunately, the two investigations reached opposite conclusions, leaving the question unresolved. [150,151]

Recently, Lopez-Rios et al., raised the possibility that false-positive results could be amplified from common laboratory vectors which contain one or more of SV40 viral DNA elements.[152] Further, Manfredi et al., have failed to detect SV40 sequences in their tumor specimens.[151] These authors call into question all previous studies that have used PCR methods to detect SV40 in human tumors, because they were conducted with primers that can amplify DNA present in common laboratory plasmids. The issue of possible laboratory contamination of specimens is not a new argument. It should be pointed out that, in the recent years, rigorous precautions have been taken in most studies. It is reasonable to suppose that, if SV40-based plasmid contamination occurred during sample processing, it would affect a very large number of samples and frequency of SV40 detection by PCR in human tumors would be higher than that reported. In this context, it should borne in mind that in recent investigations SV40 Tag mRNA and/or Tag protein have been detected both in fresh and paraffin-embedded tissues. Moreover, in human specimens sequence variability in the SV40 Tag C-terminal coding region and regulatory region have been detected, thus adding additional support to the circulation of different SV40 strains in humans. Indeed, many reports indicate that different SV40 strains and variants are distributed throughout the human population and consequently in human specimens. Variations in the Tag-C gene region have frequently been detected in human tumor associated sequences. The Tag-C sequence was shown to be stable during the tissue culture passage of SV40 isolates. In contrast, the SV40 regulatory region may contain large insertions, deletions or duplications, and rearrangements have been observed to occur within individual infected monkeys and during the passage of SV40 in certain cultured cells. Several SV40 genotypes from monkeys, contaminated vaccines and humans, are common to different population sources. Interestingly, studies

based on known isolates and genomic fragments indicate that monkeys, vaccine and human populations contain SV40 genotypes both with one and two 72-bp repeats in the enhancer domain of the regulatory region. Studies derived from the United States mainly detected one 72-bp repeat in human tumor tissues although SV40 regulatory region sequences with two 72-bp repeats were also detected in human osteosarcoma and mesothelioma samples. Moreover, SV40 wild type strain 776, which has two 72-bp repeats in the enhancer domain of the regulatory region, was the main representative among the different SV40 strains detected in kidney, urine and blood samples of an American group consisting of normal people and patients affected by focal segmental glomerulosclerosis. In a recent study carried out in Italy, SV40 sequences were detected in peripheral blood lymphocytes from Caucasian organ donors of different ages. Interestingly, the SV40 regulatory region detected in these human specimens showed DNA sequence variability. This result confirms and extends previous data on the circulation of different SV40 strains and variants in different populations. In addition, the presence of SV40 sequences in people born before and after the introduction of SV40-contaminated vaccine suggests that (i) SV40 is spreading by horizontal infection and probably; (ii) other unidentified sources of SV40 infection may exist.[153-155] It has been shown that some vaccine-derived SV40 genotypes overlap with those detected in monkey and in human populations, supporting the hypothesis that contaminated vaccines may play a role in the introduction of SV40 into the human population. Moreover, it should be noted that viral isolates from humans with one 72-bp in the regulatory region have not diverged from monkey isolates, showing that adaptation is not essential for viral survival in humans.[156]

As mentioned above, SV40 sequences in human tumors were often detected at low level, less than one genome equivalent per cell, while the Tag expression when detected was revealed only in a fraction of tumor cells. These data obtained with human specimens differ from the results from rodent cells, where the SV40 sequences are present in each cell and the SV40 Tag must be continuously expressed to transform the cell and then to maintain transformation. In order to understand, at least in part, these differences between human and rodent models, some considerations should be made. It is well established that the SV40 Tag induces chromosome aberrations which are likely to affect the functions of genes involved in tumorigenesis, such as oncogenes, tumor

suppressor and DNA repair genes. Once chromosomal damage has been triggered in tumors and chromosomal aberrations have reached a threshold, genomic instability ensues, due to the functional alteration of DNA repair genes, leading to more genetic lesions and tumor progression. This process does not need the long-period maintenance of the original oncogenic agent that caused the injury and initiated the tumorigenesis process. Therefore, in human cells SV40 could initiate the tumorigenic process by hitting the cell genome, then it could become dispensable and lost in the progression phase of the tumor, when the accumulation of genetic alterations renders the presence of viral transforming functions unnecessary. Immunoselection may even be exerted against persistently SV40-infected cells, while genetically mutated and uninfected cells may have a proliferative advantage and become the prevalent population in the tumor tissue. This "hit and run" mechanism was originally proposed to explain transformation of human cells by the mutagenic herpesviruses, and has been recently suggested to be effective in colorectal carcinogenesis associated with JCV, a polyomavirus closely related to SV40. On the contrary, in SV40-transformed rodent cells, SV40 sequences are not lost. This difference mainly depends on rodent cells, which are non-permissive to SV40 multiplication. Therefore, the input viral DNA is integrated into the cell genome. Since many human cells are semi-permissive to SV40 infection, the viral genome replicates poorly in these cells. Consequently, the few replicated DNA molecules remain in the episomal state, or are even lost, in a fraction of the cells. Another mechanism of transformation, the paracrine mechanism, exerted by the SV40 Tag has been revealed in murine and canine cells. Indeed, it has been shown that the insulin-like growth factor type I (IGF-I) and hepatocyte growth factor (HGF) are secreted in SV40-positive cells and may stimulate proliferation/transformation. Similarly, in human mesothelial cells SV40 Tag activates an autocrine/paracrine loop, involving the hepatocyte growth factor (HGF) and its cellular receptor, which is the product of the oncogene c-met, as well as the vascular endothelial growth factor (VEGF) and its cellular receptor VEGFR. HGF and VEGF, released from SV40-positive human cells, bind their receptors in neighboring and distant SV40-positive and SV40-negative cells, driving them into proliferation and tumorigenesis. In this human cell model only one cell out of 100/1000 needs to express the Tag to transform all the cells of the monolayer.[157-159] Suggestive data are available on the role of SV40 Tag in

the pathogenesis of human mesothelioma: (i) its ability to bind in vivo p53 and RB family proteins in human mesothelioma samples; (ii) activation of Notch-1, a gene promoting cell cycle progression and cell proliferation, in primary human mesothelial cells; (iii) induction of apoptosis in mesothelioma cells transfected with antisense DNA to the SV40 early region gene; (iv) the presence of SV40 Tag-specific cytotoxic T lymphocytes in sera of patients affected by mesothelioma; and (v) the poorer prognosis of mesotheliomas harboring SV40 early region sequences compared to SV40-negative mesotheliomas. Moreover, mesothelial cells are particularly susceptible to infection and transformation by SV40. Asbestos, which is the main cause of human mesothelioma, cooperates with SV40 in transformation of murine cells as well as of human fibroblasts and mesothelial cells, suggesting that SV40 and asbestos may be co-carcinogens in the onset of the mesothelioma. Fluorescent in situ hybridization analysis has indicated that the RB and cyclin E/CDK2 genes undergo the same type of deregulation during the cell cycle in asbestos treated and SV40-transformed human mesothelial cells as well as in mesothelioma cells. Recently, it has been shown that SV40 tumor antigens induce telomerase activity in human mesothelial cells, but not in human fibroblasts, suggesting that both SV40 oncoproteins specifically participate in the immortalization of mesothelial cells during mesothelioma development.[160-162]

Chapter 2

Objective

2.1 Objective

Simian virus 40 (SV40) was characterized as a transforming and tumorigenic viral agent; it transforms different types of animal and human cells, and when inoculated by different routes in experimental animals, it induces bone and brain tumors, mesotheliomas and lymphoproliferative disorders, including lymphocytic leukemia, histiocytic lymphomas and rarely B-cell lymphomas. SV40 footprints in humans have been found to be associated at high prevalence with specific human tumors of the same types as are induced by SV40 in rodents, mostly using PCR analysis. SV40 sequences were also detected, although at lower prevalence, in blood samples from healthy donors. In SV40-positive human specimens, the viral DNA load is usually at a low level, suggesting that human cells are only in part permissive for its multiplication. Cell transformation is induced by Tag and tag, which display multiple functions. Contrasting

reports have appeared in the literature on the presence of SV40 footprints in humans and its association, as a possible etiologic cofactor, with specific human tumors. As a consequence of these results, considerable debate has developed in the scientific community. The problems related to SV40 infection in the human population and its contribution to human cancer have been summarized in an evaluation by the "Immunization Safety Review Committee" established by the Institute of Medicine of the National Academies. The Committee stated that "the evidence is inadequate to accept or reject a causal relationship between SV40-containing polio vaccines and cancer". In fact, epidemiological studies conducted in the past were flawed by the difficulty in establishing which individuals received contaminated vaccines, in determining the dosage of infectious SV40 present in different lots of vaccine, due to formalin inactivation of the poliovirus which may have variably affected SV40 infectivity, and finally, in observing large cohorts of subjects for several decades after virus exposure to monitor for cancer development. The Committee concluded that "the biological evidence is strong that SV40 is a transforming virus, but it is of moderate strength that SV40 exposure from polio vaccine is related to SV40 infection in humans and that SV40 exposure could lead to cancer in humans under natural conditions". The Committee also recommended the development of specific and sensitive serologic tests for SV40 and the use of standardized techniques which should be accepted and shared by all laboratories involved in SV40 detection.[163] Immunological assays for the identification of SV40-seropositive healthy individuals, oncologic patients and serum antibody reactivity to SV40 Tag and VP antigens are of paramount importance to reveal the prevalence of SV40 infection in humans and to study the association of SV40 with specific human tumors.

In this investigation, serum samples from healthy donors and oncologic patients were analyzed for exposure to SV40 infection by indirect ELISA assays, employing synthetic peptides from early and late SV40 specific regions.

Chapter 3

Materials and methods

3.1 Human samples

A total of 1,837 serum samples were collected from different healthy individuals and oncologic patients in different Institutions in Italy. Sera, belonging to our collection, were taken from subjects and were obtained from discarded laboratory analysis samples during the years 2002-2009. Anonymously collected sera were coded with indications of age and gender only.

The project was approved by the local ethical Committees. The different cohorts of normal individuals were composed of (i) 316 children and adolescents, (ii) 960 adults, healthy blood donors, (iii) 94 pregnant woman, and (iv) 90 healthy workers, previously exposed to occupational asbestos. In addition, serum samples were taken from oncologic patients: 95, malignant pleural mesothelioma; 47, osteosarcoma; 44, glioblastoma; 89, non-Hodgkin lymphoma; 38, breast cancer; and 64, undifferentiated

nasopharyngeal carcinoma. Different cohorts were homogeneously clustered by age, gender and geographic region of Italy (Figure 3.1).

Figure 3.1 Different cohorts distribution. Different cohorts were homogeneously clustered by age, gender and geographic region of Italy.

3.2 Synthetic peptides

Computer assisted analyses allowed us to select specific SV40 peptides/epitopes of early and late viral regions by comparing Tag amino acids (a.a.) and three capsid

proteins, VP 1-3 from SV40, with the a.a. of the BKV and JCV polyomaviruses, which are highly homologue to SV40, as well as with other polyomaviruses with less homology (Figure 3.2a and 3.2b) (web site, http://blast.ncbi.nlm.nih.gov). The two selected SV40 peptides early region map in the Tag carboxyl-terminal domain, whereas the two peptides of the late viral region belong to VP1/VP3 capsid proteins (Figure 3.2a). The amino acid sequences of the four peptides are as follows:

B: NPDEHQKGLSKSLAAEKQFTDDSP

B: ASN-PRO-ASP-GLU-HIS-GLN-LYS-GLY-LEU-SER-LYS-SER-LEU-ALA-ALA-GLU-LYS-GLN-PHE-THR-ASP-ASP-SER-PRO

(60 - 83)

C: IQNDIPRLTSQELERRTQRYLRD

C: ILE-GLN-ASN-ASP-ILE-PRO-ARG-LEU-THR-SER-GLN-GLU-LEU-GLU-ARG-ARG-THR-GLN-ARG-TYR-LEU-ARG-ASP

(50 - 72)

A: KGSCPGAAPKKPKEPV

A: LYS-GLY-SER-CYS-PRO-GLY-ALA-ALA-PRO-LYS-LYS-PRO-LYS-GLU-PRO VAL

(9 - 24)

D: DSIQQVTERWEAQSQSPNVQSG

D: ASP-SER-ILE-GLN-GLN-VAL-THR-GLU-ARG-TRP-GLU-ALA-GLN-SER-GLN SER-PRO-ASN-VAL-GLN-SER-GLY

(135 - 156)

The a.a. acid residues of the two specific SV40 Tag epitopes, known as Tag A and Tag D, show little or no homology with the corresponding Tag region of BKV and JCV (Figure 3.2b). Similarly, the two specific SV40 VP epitopes of the late region, known as VP B and VP C, show no/low homology with the VPs of BKV and JCV (Figure 3.2b). The synthetic peptides were synthesized by standard procedures and were purchased by UFPeptides s.r.l., Ferrara, Italy.

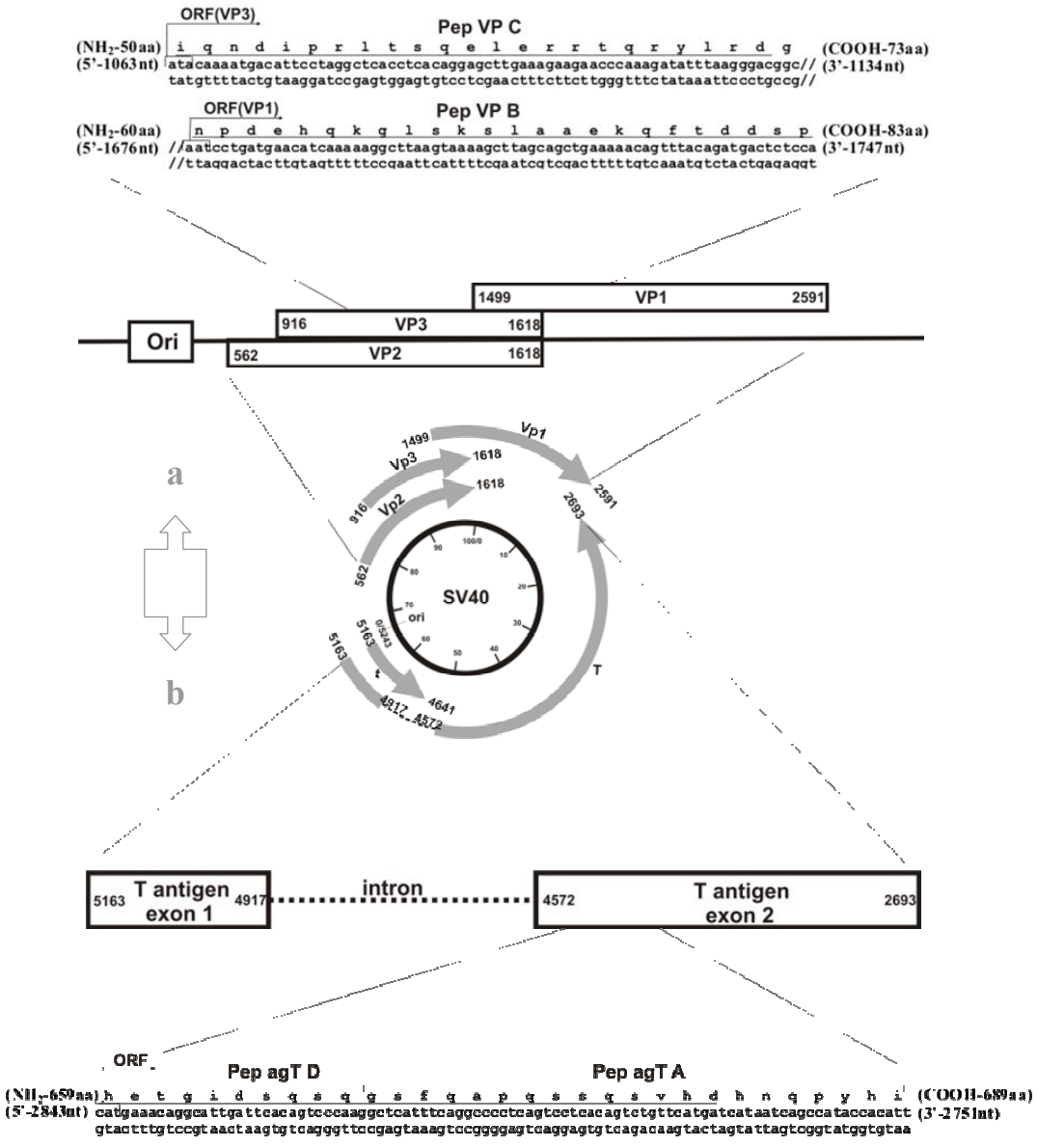


Figure 3.2 SV40 peptides. (a, b) Specific SV40 peptides/epitopes of early and late viral regions by comparing Tag a.a. and three capsid proteins, VP 1-3 from SV40, with the a.a. of BKV and JCV polyomaviruses, which are highly homologue to SV40, as well as with other polyomaviruses with less homology. **(a)** The two selected SV40 peptides early region map in the Tag carboxyl-terminal domain, whereas the two peptides of the late viral region belong to VP1/VP3 capsid proteins. **(b)** The amino acid residues of the two specific SV40 Tag epitopes, known as Tag A and Tag D, show little or no homology with the corresponding Tag region of BKV and JCV. Similarly, the two specific SV40 VP epitopes of the late region, known as VP B and VP C, show no/low homology with the VPs of BKV and JCV.

3.3 Cell lines

Normal African Green Monkey Kidney Epithelial Cells (Vero), Normal African Green Monkey Kidney Fibroblast Cells (CV-1), and Human Brain Astroglia, SV40 transformed (SVG p12) were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA, USA).

■ **Vero cell line**

The Vero cell line was established from the cells isolated from a kidney harvested from Cercopithecus aethiops (African green monkey) in 1962 by Yosihiro Yasumura. The name "Vero" chosen by Yasumura means "truth" in Esperanto. The etymology is of interest since "Vero" is also an acronym formed from the constituent parts "verda" (meaning green in Esperanto) and "reno" (kidney): hence African green monkey kidney. Cells exhibit the morphology epithelial, grow adherently to glass or plastic surfaces. Vero cells are sensitive to infection with SV-40, SV-5, measles, arboviruses, reoviruses, rubella, simian adenoviruses, polioviruses, influenza viruses, parainfluenza viruses, respiratory syncytial viruses, vaccinia, and others. This is a cell line with the hypodiploid chromosome count: the modal chromosome number was 58 occurring in 66% of cells. In most cells, over 50% of the chromosomes in each cell complement belonged to structurally altered marker chromosomes. Normal A3, A4, B4, and B5 were absent; B2, B3 and B7 were occasionally paired; and B9, C1 and C5 were mostly paired. The rate of cells with higher ploidies was 1.7%. Other chromosomes were mostly present in single copy. Vero cells were grown in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS). They were maintained in the same medium supplemented with 2% FBS (Figure 3.3a).

■ **CV-1 cell line**

The CV-1 cell line was initiated in March of 1964 by F.C. Jensen and his colleagues with a tissue section excised from the kidney of a normal adult male African green monkey (Cercopithecus aethiops). Cells of the CV-1 line exhibit the morphology of fibroblasts, grow adherently to glass or plastic surfaces. CV-1 cells are susceptible to several viruses, including poliovirus 1, herpes simplex, SV40, California encephalitis, and both Eastern and Western equine encephalitis. The modal chromosome number was 60, occurring in 48% of cells, and the rate of polyploidy was at 4.4%. Only a few markers were found., of these M1, a probable deleted N11, was found in all cells

examined; M3 of unknown origin was in some cells; and the remaining 2 to 3 others of unknown origins were found only once., N11 was uniformly single copied, and N16 was also single copied in most cells. Both X and Y chromosomes were also detected in every cell. CV-1 cells were grown in DMEM supplemented with 10% FBS. They were maintained, after infection with SV40, in the same medium supplemented with 1% FBS (Figure 3.3b).

■ **SVG p12 cell line**

The SVG p12 cell line was established by transfecting cultured human fetal glial cells from brain material dissected from 8 to 12 week old embryos with DNA from an ori-mutant of SV40. The cells express SV40 Tag and are sensitive to infection with JCV. They were grown in Minimum Essential Medium Eagle (EMEM) supplemented with 10% FBS and sodium pyruvate 1 mM. They were maintained, after infection with JCV, in the same medium supplemented with 2% FBS (Figure 3.3c).

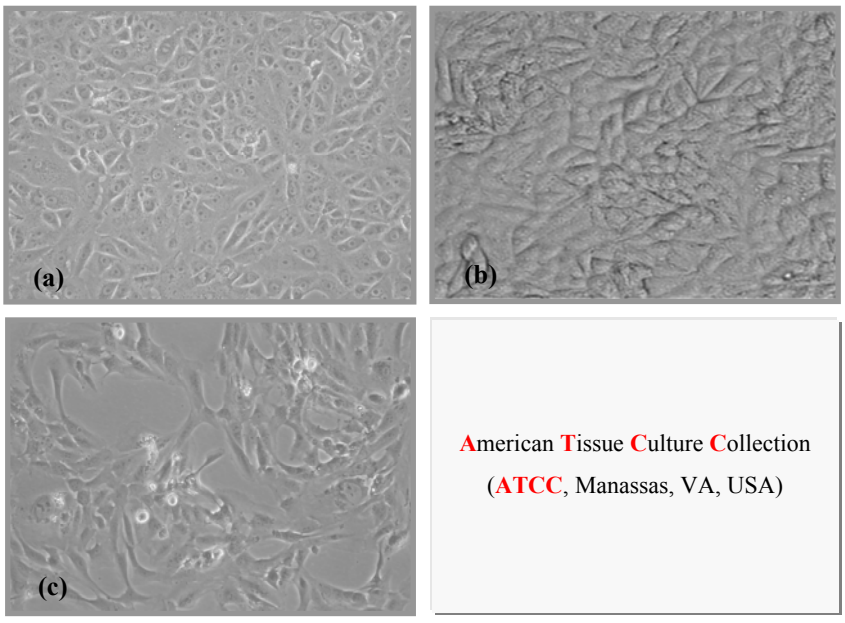


Figure 3.3 Cell lines. (a) Normal African Green Monkey Kidney Epithelial Cells (Vero). **(b)** Normal African Green Monkey Kidney Fibroblast Cells (CV-1). **(c)** Human brain astroglia, SV40 transformed (SVG p12).

3.4 Viruses

BKV was kindly supplied by Dr S.D. Gardner, while SV40 (776 strain) and JCV (MED-4 strain) were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA, USA).

3.5 Preparation of virus stocks

SV40 and BKV preparations obtained by high multiplicity of infection may contain variant and defective viruses (evolutionary variants). They arise by rearrangement, reiteration and deletion of viral DNA sequence and by substitution of viral DNA with host cell DNA. By maintaining the viral origin of replication, defective viruses are perfectly able to replicate in the presence of wild-type virus and to form progeny particles.[164] The following procedure, which is suitable for both SV40 and BKV. For SV40 use confluent cultures of CV-1 cells, for BKV confluent cultures of Vero cells.

■ **Protocol**

1. Withdraw medium and discard.
2. Infect cultures confluent cells (75-80%).
3. Add 1 ml of the suspension viral on each dish (Table 3.1).

Virus	Viral stock (pfu/ml)	Viral stock (µl)	DMEM (µl)
SV40	$1 \cdot 10^7$	5 (a)	995
BKV	$3.84 \cdot 10^8$ (*)	5 (b)	995

(*) $3{,}84 \cdot 10^8$ pfu = $1{,}28 \cdot 10^2$ HAU (1 HAU = $3 \cdot 10^6$)[601] (a) 5 µl = $5 \cdot 10^4$ pfu/ml (b) 5 µl = $1{,}92 \cdot 10^6$ pfu/ml

Table 3.1 Viral concentration

4. Incubate the cultures at 37 °C for 90 min for adsorption of the virus to the cells. During this period rock the dishes from time to time to redistribute the virus suspension.

5. After the adsorption period, cover the cultures with 5 ml of pre-warmed DMEM containing 1% FBS.
6. Incubate the cultures for 14-18 days until clear signs of cell lysis are observed (see Figure 3.6).

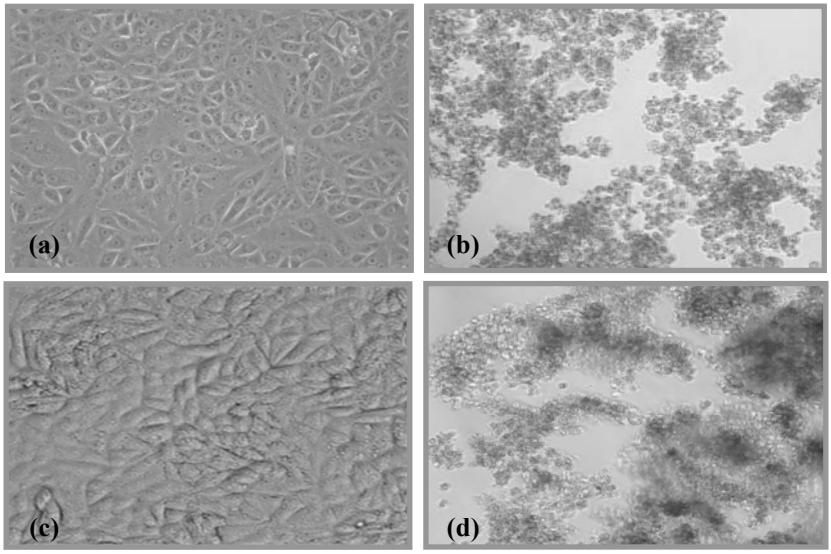


Figure 3.6 Cells infection. **(a)** Infection of Vero cells with BKV after 1 day: cell intact layer. **(b)** Infection of Vero cells with BKV after 15 day: disintegration of cell layer. **(c)** Infection of CV-1 cells with SV40 after 1 day: cell intact layer. **(d)** Infection of CV-1 cells with SV40 after 14 day: disintegration of cell layer.

7. Scrape whatever remains of the monolayer with a sterile silicone rubber and collect the culture medium and the cell debris into a small flask.
8. Freeze and thaw thrice, then sonicate to disintegrate the cell debris (3 cycles of 1 min).
9. Centrifugation in a sterile tube at 8,000 g for 10 min at 4 °C, pour the supernatants into a sterile flask and resuspend the pellet using 1/20 of the volume of the supernatants. Sonicate the cell debris and centrifuge at 10,000 g for 20 min. add the supernatant to the flask containing the first supernatant. This is now the virus stock suspension. Treat the cell debris with clorox or 5% hypochlorite and discard.

3.6 Purification of SV40 and BKV

SV40 and BKV are not sensitive to lipid solvents such as chloroform, nor to CsCl solutions, which can therefore be used during the purification of the viruses. The method given is suitable for both SV40 and BKV.

- **Protocol**

1. Further concentration was achieved by pelleting the virus at 80,000 g at 4 °C.
2. The pellet was resuspended in a 2 ml of 10 mM Tris-HCl pH 7.5. The suspension should be homogeneous and without clumps. Transfer the suspension to an ultracentrifuge tube (e.g., Beckman rotor SW60 or SW50.1) and adjust it with buffer to 2,5 g by weighing. Add 1,20 g of CsCl and make a homogeneous solution by gentle pipetting. The density of the solution should be 1.30-1.33 g/ml and should be checked by measuring the refractive index. Fill the centrifuge tube with paraffin oil and equilibrate your tubes and balance tubes.
3. Centrifuge at 35,000 g for about 20 h at 20 °C. Virus particles band at a density of 1.33 g/ml; empty capsids, which are always present in SV40 and BK virus preparations, band at a density of 1.29 g/ml. Cell debris is on top of the gradient. Often the two bands of virus particles and empty capsids can be see by eye.
4. Collect the gradient by dividing it into 16-20 fractions, i.e., about 4-6 drops per fraction. There are several ways of doing this. We use a simple tube holder with a screw cap. The bottom of the tube is pierced with a hypodermic needle and the dripping out of the gradient is controlled by placing the forefinger on the opening of the screw cap.
5. Identify the virus-containing fractions with of the following method: dilute each fraction to 1 ml with 10 mM Tris-HCl pH 7.5 and read the optical density at 260 nm (thereafter the virus can be concentrated again by centrifugation).
6. Pool the fractions containing the virus and dialyse in the cold against 10 mM Tris-HCl pH 7.5 or 1 mM sodium phosphate pH 6.8. Alternatvely, you may remove CsCl by diluting the virus with buffer and by two subsequent sedimentations of the virus particles (80,000 g, 3 h at 4 °C).
7. If the virus is used for infecting cell cultures do not forget to re-adjust the salt concentration to isotonic conditions. Sterilise the virus suspension by filtration through an 0.2 mm membrane filter.

3.6.1 Titering SV40 by Plaque Assay

- **Outline.** A plaque is a small area where the cell monolayer has destroyed by virus. The principle of the plaque assay is to infect the monolayer with virus diluted to contain only a few infectious units, then to cover the monolayer with agar which prevents progeny virus from diffusing all over the plate. Virus from a single lysed cell can still infect adjacent cells, giving rise after several cycles to a plaque.
- **Protocol**

1. Prepare serial dilutions of the SV40 stock in DMEM-1% FBS and vortex vigorously. Note: Precise serial dilution is a key step in SV40 stock titering. It is critical that each dilution be thoroughly mixed before it is used to make the subsequent dilutions. It is also important to make the serial dilutions immediately before they are used because the virus in very dilute solutions tends to be less stable.
2. Use 1 ml of each dilution to infect 6-cm dishes of freshly confluent dishes of CV-1 after removing the old medium. Include a dish with 1 ml of DMEM-1% FBS without virus as mock control.
3. Incubated at 37 °C for 2 h with rocking at 15-min intervals.
4. After this 2-h period, remove the 1 ml of DMEM and overlay the monolayers with 4 ml of a fresh mixture containing 1 part melted 1.8% Bacto-Agar cooled to 45 °C and 1 part 2X modified Eagle's medium (without phenol red) with 10% FBS at 37°C. Allow the overlay to harden at room temperature for approx 15 min before returning dishes to the incubator.
5. Add an additional 3 ml of this mixture every 3 day with the exception of the sixth day, when neutral red should be added to the overlay mixture at a final concentration of 50 μg/ml, in order to visualize the plaques.
6. Plaques should be visible and can be counted on the eighth day and beyond. The number of plaques times the inverse of the dilution factor gives the titer of the virus stock in pfu/mL.

- **Titer**

  The purified SV40 preparation had an infectious titre of $5x10^7$ plaque forming units (pfu)/ml.

3.6.2 Titering BKV by Hemagglutination Assay

In addition to direct assays for infectious BKV, a measure of the quantity of virus in a pool can be obtained rapidly and simply by titration of the hemagglutinin present provided there is a sufficiently high titer of virus; it has been estimated that 1 HAU is equivalent to $3 \cdot 10^6$ virus particles/mL, which includes both complete particles and empty capsids.[165-167]

- **Outline.** All of the polyomaviruses, with the exception of SV40, hemagglutinate red blood cells (RBCs). In a hemagglutination assay, RBCs are added to a dilution series of virus in a U-bottomed microtiter dish. The RBCs sediment to the bottom of the well and form a red "button." In the presence of BKV, however, the virus will bind to sialic acid residues on the surface of the RBCs, forming a network of RBCs and virus that prevents the formation of the red button; a smooth "carpet" is formed instead.

- **Protocol**

1. Obtain at least 5 mL of human type O, Rh+ blood. Wash the whole blood three times with PBS, spinning at 1000 *g* for 10 min at room temperature each time. After each wash, carefully remove the whitish/yellow layer of leukocytes that form on top of the RBC pellet. After the washes, resuspend the RBCs in 20 mL of PBS for use and storage. Store at 4°C. Under these conditions, RBCs remain usable for about 10 days.
2. The hemagglutination assay requires a 0.5% suspension of red blood cells diluted in PBS. This can be prepared by pelleting the RBCs, discarding the supernatant and resuspending the pellet to 0.5% v/v in PBS.
3. Prepare twofold serial dilutions of the virus stock in PBS. This can be conveniently done by adding 50 µL of PBS to each well of a 96-well U-bottom plate and adding 50 µL of sample to the first well and diluting it 1:2 across the plate. Include a positive control (virus with a known titer) and a negative control (no virus).
4. Add 50 µL of the 0.5% RBC solution to each well. Shake the plate very gently to mix. Incubate the plate for 4 h at 4°C undisturbed.

- **Titer**

The purified BKV preparation had an infectious titre of $5 \cdot 10^3$ hemagglutination units (HAU.)/ml, corresponding to $1.5 \cdot 10^{10}$ physical particles/ml (1 HAU = $3 \cdot 10^6$).

3.7 Control immune sera

Hyperimmune sera against SV40 and BKV were obtained in rabbits inoculated with purified viral stocks as previously performed. The serum against JCV was kindly provided by Dr. Major, NIH, Bethesda (MD), USA.

3.7.1 Polyclonal antibody production

- **Protocol**

1. Animals

Rabbits are the species used for polyclonal antibody production, principally because they are of a size capable of producing adequate amounts of antibodies, have a relatively long life span, and are relatively easy to handle.

a. Male New Zealand white rabbits with an average body weight of 2.5 ± 0.2 kg were used in this study.
b. The rabbits have access to commercial rabbit food and water ad libitum and the diet is supplemented with hay three times a week and carrots once each week.
c. All rabbits are acclimatized to their new surroundings for a minimum of 2 weeks prior to any procedures.

2. Administration of antigens

a. The antigen was provided in a stable emulsion ready for injection to a maximum volume of 1 ml. The emulsion was been passed easily through an 18 gauge needle.
b. Initial immunizations consist of antigen in either Freund's Complete Adjuvant (FCA) given subcutaneously (sc) in up to four sites (2 sites at the neck and 2 sites at the flank) with a maximum of 0.25 ml per site, since a maximum of 1 ml of antigen plus adjuvant may be injected, divided among 4 sites. FCA is composed of 1mg/ml heat killed, dried Mycobacterium species added to mineral oil (Bayol F) at 85% and a detergent (Arlacel Ai mannide monooleate) at 10-15%.
c. If CFA was used for initial immunization, subsequent immunizations consist of Freund's Incomplete Adjuvant (FIA). 500 µl of BKV, $7.5 \cdot 10^9$ pfu, diluted in 500 µl of emulsified Freund's complete adjuvant was employed for the first injection and then twice with the same amount of virus in Freund's incomplete adjuvant. As regards SV40, 500 µl, $2.5 \cdot 10^7$ pfu, were diluted with the same amount of FCA and FIA.

3. Serum collection

a. The venous blood of the rabbit was collected into a tube, containing a protease inhibitor solution.

b. The tube was inverted slowly 8-10 times to mix protease inhibitors coagulant with the blood. The tube was maintained at room temperature (22 °C) for 60 minute and it was centrifuged at 1400-2000 g for 10 min at 4 °C. Within 30 minutes of centrifugation, was transfered the supernatant (serum) in 1-ml aliquots to pre-labeled Fisherbrand 4-mL self-standing cryovials.

c. The serum was placed aliquots freeze aliquots at -80 °C until used.

4. Serum titration

a. The immune serum anti-BKV was titrated by the hemagglutination inhibition (HAI) test employing human erythrocytes from the 0, Rh+ group. The endpoint antibody titer of the BKV antiserum, determined by serial dilution, was 12,800 HAU, as will be shown in section 3.9.2.

b. Anti SV40 serum was titred by neutralization. In brief, serial dilution of titred SV40 stock (10^7 pfu./ml) was mixed with heat-inactivated immune rabbit serum. Specifically, 1 ml of the solution containing SV40 ($5x10^4$ pfu in 5 µl and 995 µl of serum) was incubated for 30 min. at 37 °C, and then used as inoculum in permissive CV-1 monkey kidney cells grown in 25 cm^2 flasks ($3x10^6$ cells). After 2 hours adsorbtion, the inoculum was discarded and cells overlaid with DMEM with 1% FBS. The cytopathic effect (CPE) due to SV40 multiplication was inspected from day 10 to day 15. SV40 hyperimmune rabbit serum was able to completely inhibit CPE in CV-1 cells. Negative and positive controls were uninfected and SV40-infected CV-1 cells, respectively.

3.8 **E**nzyme **L**inked **I**mmuno**S**orbent **A**ssay (ELISA)

The indirect ELISA test was developed to detect specific antibodies against SV40 in human sera. ELISA plates were coated with synthetic peptides corresponding to specific SV40 epitopes encoded by early and late viral DNA regions. Specifically, two synthetic peptides were from the main viral transforming protein, known Tag, which is encoded by the early region of SV40 genome, while two other synthetic peptides were from viral capsid proteins VP1/VP3 of the late region of SV40 (Figure 3.2a and 3.2b).

3.8.1 Reaction scheme

I-Ag	+	Ab	+	Anti-Ab*E	+	S	→	Read
W		W		W				

I- = Microplate wells; Ag = Peptide adsorbed to wells; Ab = Serum (primary antibody); Anti-Ab*E = Secondary antibody conjugated with peroxidase; S = Substrate (ABTS); + = Incubation; W = Washing (Figure 3.7).

3.8.2 Basic of Assay

The basic of this assay is to titrate antibodies that have reacted with an antigen by using an antispecies conjugate. The indirect aspect, therefore, refers to the fact that the specific antiserum against the antigen is not labeled with an enzyme, but a second antibody specific for the particular species in which the first antibody was produced is labeled.

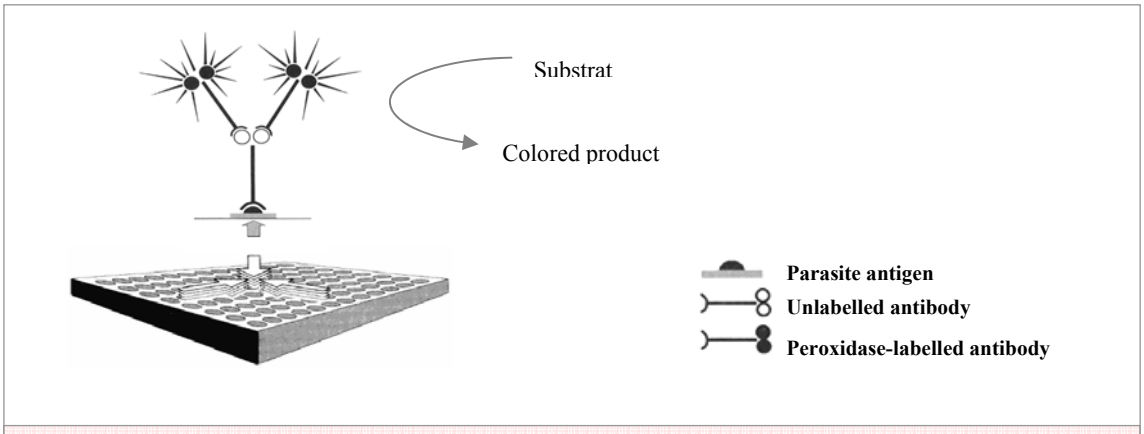


Figure 3.7 Indirect ELISA.

■ Materials

96 flat bottom wells (Nunc-immuno plate PolySorp, CelBio, Milan, Italy)

Coating Buffer (Candor Bioscience, Weissensberg, Germany)

Washing Buffer (Candor Bioscience, Weissensberg, Germany)

Blocking Solution (Candor Bioscience, Weissensberg, Germany)

Low Cross-Buffer (Candor Bioscience, Weissensberg, Germany)

Primary antibody: human serum samples

Secondary antibody: (1) Goat anti-Human IgG H&L chain specific peroxidase-conjugate (Calbiochem-Merck, Germany); (2) Peroxidase-labelled affinity purified antibody to human IgM ν (KPL Gaithersburg MD, USA)

2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Sigma-Aldrich, Italy)

Washing apparatus (Thermo Electron Corporation, model Wellwash 4MK2, Finland)

Spectrophotometer (Thermo Electron Corporation, model Multiskan EX, Finland)

- **Protocol**

1. *Peptide coating.* Plates, 96 flat bottom wells were coated with 5 μg of the selected peptide for each well, diluted in 100 μl of Coating Buffer. Plates were incubated at 4 °C for 16 hours allowing the peptide to completely cover the bottom well.
2. *Peptide blocking.* Plates were rinsed three times with Washing Buffer. This procedure eliminates the uncoated peptide. Blocking was made with 200 μl/well of the Blocking Solution at 37 °C for 90 min. This procedure allows the saturation of the well.
3. *Primary antibody adding.* To eliminate the residual blocking solution, plates were rinsed again 3X with the Washing Buffer in washing apparatus. Then, different wells were covered with 100 μl containing the following sera: positive-control, represented by the immune rabbit serum containing anti-SV40 antibodies, negative controls represented by the immune sera with anti-BKV and anti-JCV antibodies; and human serum samples under analysis diluted at 1:20 in the Low Cross-Buffer. Each sample was analyzed three times. In each plate, additional controls were represented by a well with the secondary antibody and two other wells void of both primary and secondary antibodies. The plate was incubated at 37 °C for 90 min.
4. *Secondary antibody adding.* After 90 min of incubation, a new three times rinsing cycle was repeated as described above. Then, the secondary antibody solution was added to each well. The solution contained a goat anti-human IgG heavy and light chain specific peroxidase-conjugate or peroxidase-labelled affinity purified antibody to human IgM ν diluted 1:10,000 in the Low Cross-Buffer. The reaction mixture was incubated at room temperature for 90 min.
5. *Dye treatment with 2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS) and spectrophotometric reading.* At the end of the incubation period, plates were rinsed three times with the washer buffer and then treated with 100 μl of ABTS solution

which reacted with the peroxidase enzyme for the color reaction. After 45 min of incubation, the colorimetric reaction was stopped with 100 µl of citric acid 0.1 M. The plate was then read at the spectrophotometer at a wavelength (λ) of 405 nm. This approach reveals the color intensity in wells where the immunocomplexes were formed, due to the presence of specific antibodies binding to the SV40 synthetic peptide/epitope, by optical density (O.D.).

6. *Cut-off determination* The cut-off, in each assay, was determined by the O.D. reading of two negative controls, added to the standard deviation, multiplied three times (+3SD). Sera with antibodies against SV40 were considered Tag- or VP-positive upon reacting to both peptides of the early or late region and when sera analyzed four times by indirect ELISA testing gave the same positive result.

$$\text{Deviance} = \sum x^2 - \frac{\left(\sum x\right)^2}{n}$$

$$\text{Variance} = S^2 = \text{Deviance} / (n - 1)$$

$$\text{Standard deviation} = \sqrt{S^2}$$

$$\textbf{Cut Off} = \bar{x} + 3 \text{ x standard deviation}$$

	1	2	3	4	5	6	7	8	9	10	11	12
A	C	C	POS	POS	NEG1	NEG1	NEG2	NEG2	NEG3	NEG3	1	1
B												
C												
D												
E												
F												
G												
H											43	43

Figure 3.8 Diagrammatic representation of plate. C = Negative control (Coating + Low cross buffer + secondary antibody + ABTS + citric acid); NEG1 – NEG3 = Human sera: negative control; POS = Polyclonal serum produced in hamster: positive control 1 – 43 = Human sera: samples.

■ Automatic ELISA assay

The ELISA assay was set up manually so that the analysis was rigorous and repeatable. Then, the same assay was transferred and repeated on the automatic ELISA processing system with the DSX instrument (Dynex Technologies Inc., VI, U.S.A.). This instrument is routinely employed in the Analysis Laboratory of the University/

Hospital, Ferrara, Italy, for many automatic ELISA tests with different clinical samples.

3.9 Cells and viruses

Viral working stocks were obtained in Vero cells infected with the SV40 776 strain or BKV Gardner strain, as described previously, whereas, JCV stock was produced in SVG A cells. SV40 stock had a title of $5 \cdot 10^7$ pfu/ml and was determined by the plaque assay as previously described. BKV and JCV stocks were titrated by measuring their hemagglutination activity for human erythrocytes, group 0, Rh+ type. BKV and JCV had a title of 10^3 HAU/ml.

3.9.1 SV40 Neutralization assay with human serum samples

Permissive CV-1 monkey kidney cells were used for the neutralization assay of SV40 infectivity.

- **Outline.** CV-1 cells were grown and propagated in DMEM supplemented with 10% FBS. Neutralization of SV40 infectivity was carried out by incubating each human serum. Then, the suspension was added to the CV-1 cell monolayer. Cultures were observed using a light microscope for the presence of CPE for 3 weeks.
- **Protocol**

1. Neutralization of SV40 infectivity was carried out by incubating each human serum, diluted at 1:20 in PBS with $5 \cdot 10^4$ pfu of SV40 at 37 °C for 30 min.
2. Withdraw medium and discard.
3. Add 1 ml of the suspension viral on the CV-1 cell monolayer for 2 h at 37 °C.
4. After the adsorption period, withdraw inoculum and discard.
5. Add warmed PBS (1-3 ml) to the dish, rinse the cells, and discard rinse.
6. Add 5 ml of the DMEM, supplemented with 1% FBS.
7. Incubate the cultures at 37 °C in a humidified atmosphere with 5% CO_2 until clear signs of cell lysis are observed.

- **Samples**

  Each sample was tested in duplicate. The neutralization assay included the following controls: (i) SV40 only in PBS, (ii) SV40 mixed with rabbit or human non-immune serum, (iii) SV40 mixed with hyperimmune rabbit serum, and (iv) cells only in PBS.

3.9.2 BKV and JCV hemagglutination-inhibition assays

The presence of antibodies to JCV and BKV was determined by a hemagglutination assay using human type O, Rh+ erythrocytes. The major capsid protein, VP1, is the predominant structural protein of the icosahedral virion particle and is responsible for attachment to cells and for erythrocyte agglutination.[605] Hemagglutination inhibition was used to measure the presence of serum antibody to JCV or BKV.

- **Outline.** The serum was treated with potassium periodate. After periodate treatment, glycerol was added. The treated serum samples were prepared as serial dilutions in PBS in microtiter wells. Each well then received an equal volume of virus and an equal volume of human type O, Rh+ erythrocytes.
- **Protocol**

1. The serum was treated with potassium periodate (0.05 M) for 30 min at room temperature to inactivate nonspecific inhibitors of erythrocyte agglutination.
2. After periodate treatment, glycerol was added to a final concentration of 1%.
3. The samples were incubated for 1 hr at 56 °C. The treated serum samples were prepared asserial 1:2 dilutions in PBS in microtiter wells.
4. Each well then received an equal volume of 4 HAU of virus and an equal volume of human type O, Rh+ erythrocytes. The highest dilution of serum that prevents agglutination, indicated as a button of red blood cells in the bottom of the well, was considered the antibody titer against the virus. The assays were performed in duplicate and repeated several times to eliminate the effects of nonspecific reactions and errors in dilutions or inconsistencies in end-point analysis.

3.10 Statistical Analysis

Statistical analyses were performed using Prism software (GraphPad, San Diego, CA). Data are presented as a percentage of the positive samples. The 95% Confidence Intervals (CI) of the percentage of positive samples are also reported. Differences among proportions were calculated by Chi-square testing for independence in the contingency tables. Small sample size was statistically analyzed using the Chi-square with Yates' correction.

Chapter 4

Results

4.1 ELISA Assay with serum samples from healthy individuals

In order to verify whether human sera contain IgG antibodies which react to SV40 antigens/peptides and to determine the diffusion of SV40 infection in humans, indirect ELISA assays were set up employing synthetic peptides which correspond to SV40 VPs and Tag epitopes (Figure 3.2). In the first series of experiments, serum samples from infants, adolescents, adults and elderly individuals, diluted at 1/20, were tested for the reactivity to two SV40 epitopes from VP1/VP3 capsid proteins, namely VP B and VP C (Figure 4.1a), while during the second step, our indirect ELISA assay was addressed to detecting IgG class serum antibodies against the SV40 Tag epitopes, known as Tag A and Tag D (Figure 4.1b). The origin and number of considered sera are summarized in table 4.1. The results obtained in words of positivity and percentage are given in tables 4.2 and 4.3.

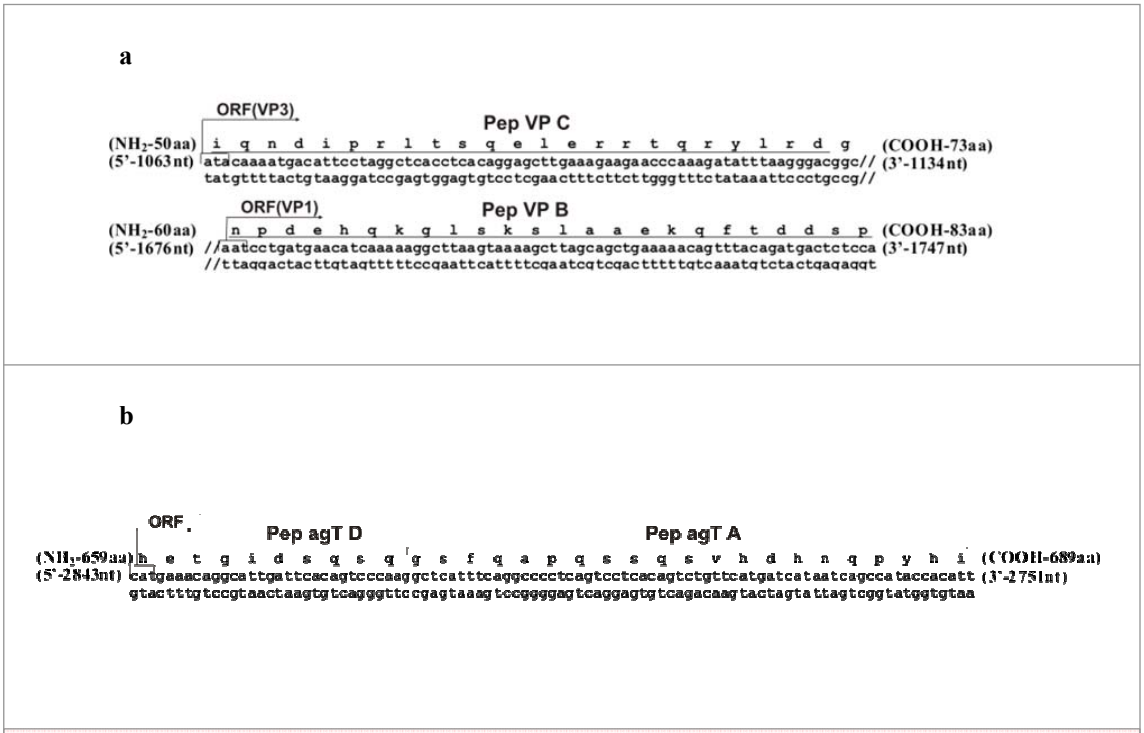


Figure 4.1 (a) The a.a. residues of the two specific SV40 VP epitopes of the late region, known as VP B and VP C. **(b)** The a.a. residues of the two specific SV40 Tag epitopes, known as Tag A and Tag D.

Origin	Age	Number samples
Ferrara University Hospital S. Anna	0-17	316
Rome Istituto Superiore di Sanità **Casale Monferrato** Casale Monferrato Hospital **Aviano** National Cancer Institute **Pisa** University Hospital **Republic of S. Marino** State Hospital	18-65	960

Table 4.1 Origin, age and number of considered sera

Age	Number samples	VP sieropositive	VP sieropositive (%)
0-1	91	11/91	12
1-17	225	42/225	19
18-50	612	120/612	20
51-65	243	30/243	12
> 65	105	24/105	23

Table 4.2 Positive samples for SV40 VPs epitopes from healthy individuals divided bracket age

Age	Number samples	Tag sieropositive	Tag sieropositive (%)
0-1	91	13/91	14
1-17	225	44/225	20
18-50	612	120/612	20
51-65	243	36/243	15
> 65	105	24/105	23

Table 4.3 Positive samples for SV40 Tag epitopes from healthy individuals divided bracket age

In the first series of experiments they deduce that in children, aged up to one year, the prevalence of SV40 antibodies IgG class was 12% (Figure 4.2A and 4.2B). Subsequently, prevalence increases with age, with an overall prevalence of 19% detected in the cohort of healthy individuals aged 1-17 years (Figure 4.2A). However, within this cohort, a prevalence of 22% was revealed in subjects aged 1-10 years (Figure 4.2B). The cohorts of healthy adults, aged 18-50 years had antibodies which were reactive to SV40 VPs, with a prevalence of 20% (Figure 4.2A). Within this cohort,

a prevalence corresponding to 23% was observed in subjects aged between 31-40 years (Figure 4.2B). The prevalence of serum antibody against SV40 VPs declines in the cohort of individuals aged 51-65 years with a percentage of 12% (Figure 4.2A). In elderly individuals aged 66-90 years, the prevalence increased to 23% (Figure 4.2A and 4.2B). During the second step, it turned out that seropositive samples to SV40 Tag peptides were the same samples, with a few exceptions, as had been found positive for the SV40 VP epitopes, employed in the previous ELISA test. Conversely, seronegative samples to SV40 VP peptides failed to react with SV40 Tag epitopes. The exceptions were negligible, and these were represented by a few serum samples which were found negative for VP while testing positive for Tag peptides. In general, the two indirect ELISA assays, with two distinct epitopes of VPs and two different peptides of the Tag gave overlapping results, thus confirming the presence of anti-SV40 Tag and anti-SV40 VP antibodies in human sera from healthy individuals (Figure 4.2A and 4.2B). Interestingly, our indirect ELISA assay for the detection of IgG class serum antibodies against the SV40 VP and Tag antigens indicated that the first appearance of antibodies against this virus was detectable in a 2 month-old child (Figure 4.2A and 4.2B, Table 4.4).

Subjects age (months)	IgM		IgG	
	VPs	Tag	VPs	Tag
2	-	-	+	+
4	-	-	+	+
6	+	+	+	+
8	+	+	+	+
8	+	+	+	+

* Indirect ELISA tests indicated the + positive and - negative sera; positive sera were those samples above the cut-off

Table 4.4 IgM and IgG class of serum antibodies reacting with SV40 Tag and VPs epitopes from five pediatric subjects aged up to 8 months.*

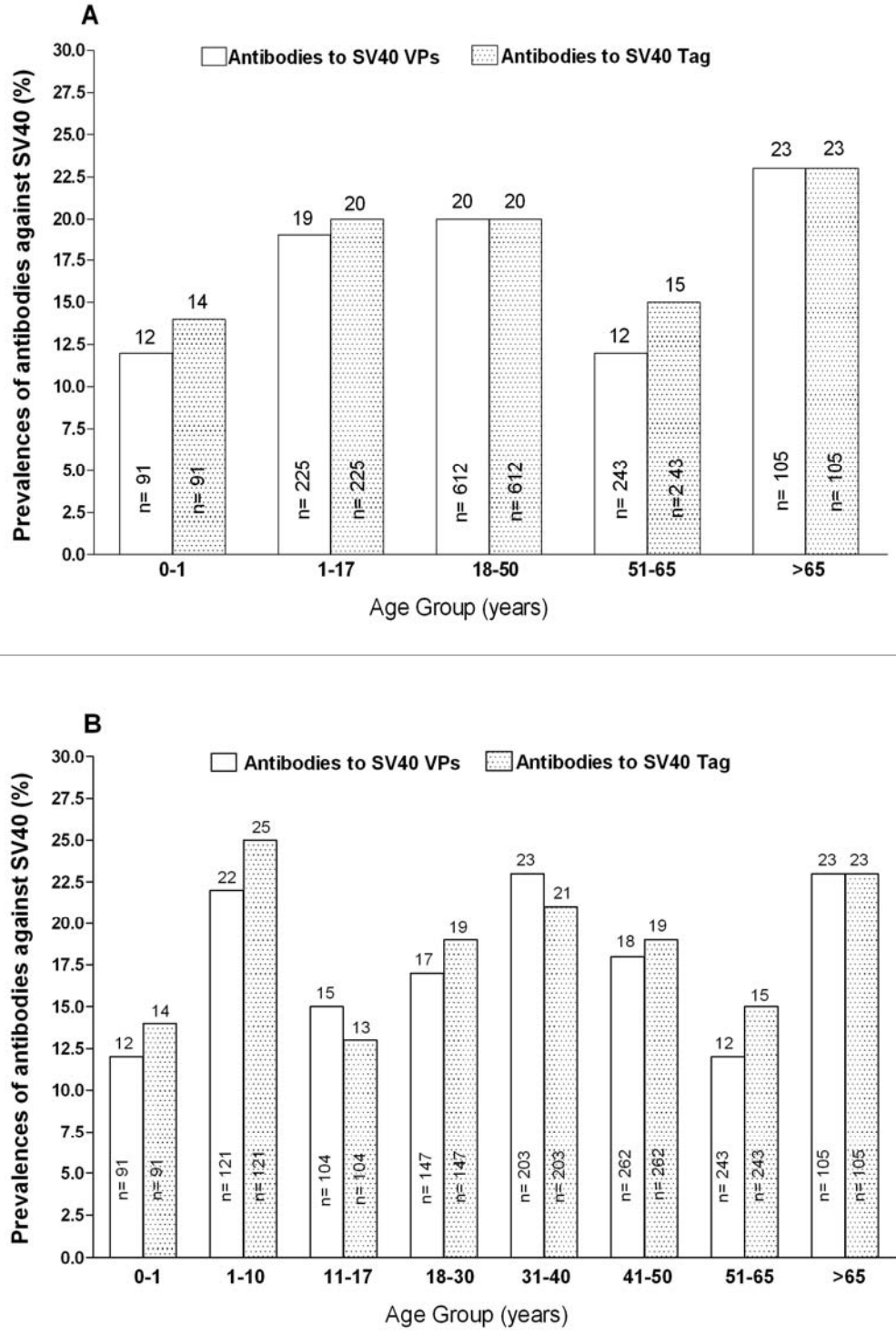


Figure 4.2 Prevalence of SV40 antibodies in serum samples from healthy subjects in different age cohorts. Prevalence is expressed as a percentage, %. The large and small year intervals are indicated in panels A and B, respectively. The number (n) of subjects in each age group is indicated inside the bar. The percentages of serum antibodies against SV40 Tag and VP are marked with dotted and white bars, respectively.

The serologic profile of individuals reacting against SV40 VP and Tag epitopes suggests that O.D. median values are 0.2 for the serum dilution at 1/20. The exception is represented by SV40-positive sera from individuals aged 1-17 years, where the median values are higher with an O.D. of 0.5 (Figure 4.3). SV40 seroprevalence of human sera was analyzed in detail, regarding age, gender and the geographic region of the individuals. ELISA data indicated that the prevalence of SV40 antibodies is similar in cohorts composed of individuals with the same age and gender, although from different regions in Italy.

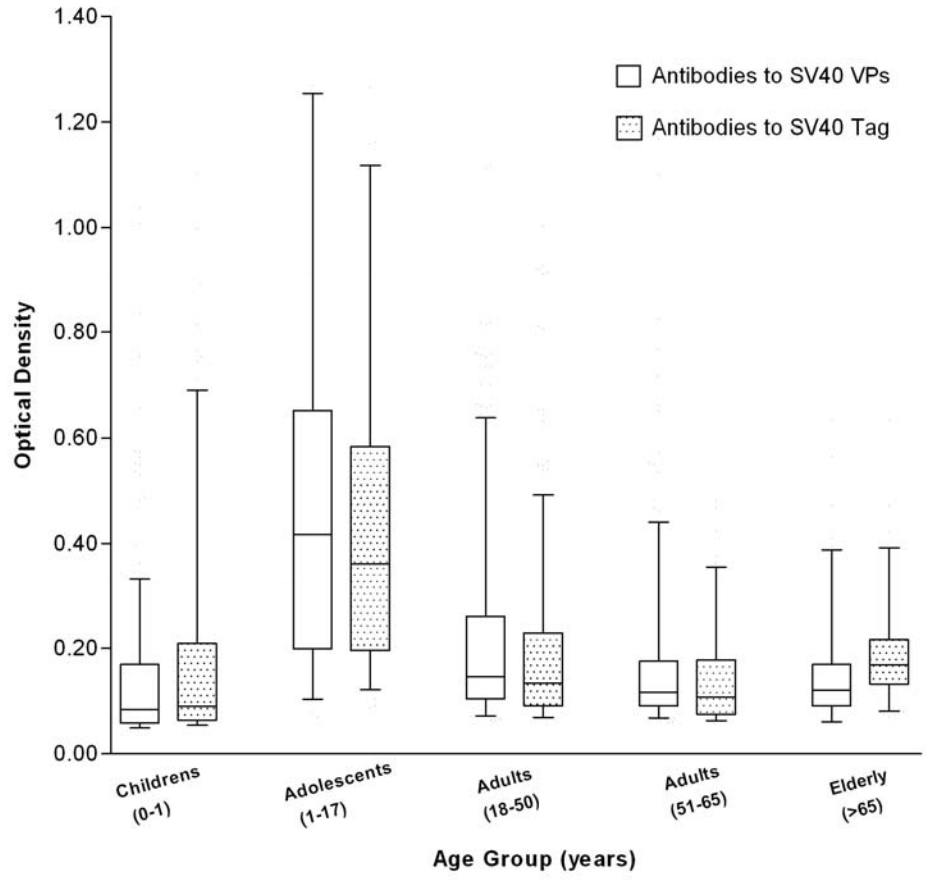


Figure 4.3 Serologic profile of 1,276 normal individuals for serum antibody reactivity to the SV40 capsid (white bar) and Tag (dotted bar) epitopes. Data are presented as values of O.D. readings at λ 405 nm of serum samples diluted at 1/20, detected in indirect ELISA assays, as described in Materials and Methods. In each box plot, the lower and upper bars represent the 5th and 95th percentiles, respectively. The line inside the box represents the median O.D level.

4.2 ELISA testing with an automatic processing system

Our ELISA assay was tested by an automatic processing system using a DSX instrument (see materials and methods). ELISA plates were manually coated with the specific peptides, while all other steps were carried out automatically with the use of the aforementioned instrument. Forty serum samples from healthy donors from a cohort covering 18-50 year-olds which were analyzed by the instrument gave the same results as previously obtained by the manually performed ELISA assay. This result is of interest since it indicates that our ELISA tests can be easily transferred to an automatic processing system in a common laboratory analysis without result variability.

4.3 Indirect ELISA assaying for IgM serum determination

In this step, the presence of IgM class antibodies reacting with SV40 epitopes, in sera from children were investigated by indirect ELISA assaying. This analysis may indicate when the first episode of SV40 seroconversion occurs in children. The analyzed sera were composed of five samples which had previously been found SV40-positive for IgG class. These samples belonged to children with ages ranging from 2-8 months who may have acquired the serum IgG antibodies from SV40-positive mothers. Our indirect ELISA assay, with four SV40 peptides for IgM determination, indicated that among these 5 sera, two samples from subjects aged 2-4 months were IgM negative, while the other three sera were IgM positive. These serum samples were obtained from children aged 6-8 months, indicating that the first episode of SV40 seroconversion occurred in our subjects when they were at least 6 months of age (Table 4.4).

4.4 ELISA assay with serum samples from oncologic patients

In our investigation, serum samples from oncologic patients affected by different neoplasms, such as osteosarcoma, glioblastoma, mesothelioma and non-Hodgkin lymphoma (NHL), were analyzed by indirect ELISA assays for the presence of antibodies against SV40 VP and Tag epitopes (Table 4.5). Indeed, several reports, mainly carried out using molecular biology techniques such as PCR methods, have indicated that SV40 is associated with these human tumors. These results suggest a role for SV40 in the onset/progression of specific human cancers. It is worth noting that other studies have not found any association between SV40 and the neoplasms

mentioned above. In order to verify whether the oncologic patients of our study had antibodies in their sera, which were reacting with specific SV40-VP and -Tag epitopes, a new round of ELISA analyses were carried out employing the same approach as described above for the sera of healthy individuals.

Origin	Patients	Age	Number samples
Ferrara University Hospital S. Anna **Modena** University Hospital	NHL	18-90	89
Bologna Rizzoli Orthopaedic Institute	Osteosarcoma	1-50	57
Verona University Hospital	Glioblastoma	18-90	34
Casale Monferrato Casale Monferrato Hospital	Mesothelioma	> 50	95
Aviano National Cancer Institute	UNPC	18-90	64
	Breast cancer	18-90	38
Pisa University Hospital	Workers ex-exsposed	> 50	90
Rome University Hospital	Pregnant women	18-50	94

Table 4.5 Origin, age and number samples from oncologic patients, workers ex-exsposed and pregnant women

In oncologic patients, results obtained in words of positivity and percentage are given in tables 4.6 and 4.7.

Neoplasm	Age	Number samples	VP sieropositive	VP sieropositive (%)
Osteosarcoma	1-50	47	25/47	53
NHL	18-90	89	41/89	46
Glioblastoma	18-90	44	15/44	34
Mesothelioma	> 50	95	25/95	26

Table 4.6 Positive samples for SV40 VPs epitopes from oncologic patients

Neoplasm	Age	Number samples	Tag sieropositive	Tag sieropositive (%)
Osteosarcoma	1-50	47	27/47	57
NHL	18-90	89	45/89	51
Glioblastoma	18-90	44	15/44	34
Mesothelioma	> 50	95	30/95	32

Table 4.7 Positive samples for SV40 Tag epitopes from oncologic patients

Two other groups of patients have been analyzed. The first group includes ex-exsposed asbestos patients and pregnant women, while the second group includes women with breast cancers (BC) and undifferentiated nasopharyngeal carcinomas (UNPC) patients. The results obtained in terms of positivity and the percentage of tables are respectively contained in tables 4.8, 4.9, 4.10, 4.11.

Patients	Age	Number samples	VP sieropositive	VP sieropositive (%)
Workers ex-exsposed	> 50	90	8/90	9
Pregnant women	18-50	94	12/94	13

Table 4.8 Positive samples for SV40 VPs epitopes from workers ex-exsposed and pregnant women

Patients	Age	Number samples	Tag sieropositive	Tag sieropositive (%)
Workers ex-exsposed	> 50	90	9/90	10
Pregnant women	18-50	94	12/94	13

Table 4.9 Positive samples for SV40 Tag epitopes from workers ex-exsposed and pregnant women

Patients	Age	Number samples	VP sieropositive	VP sieropositive (%)
BC	18-90	38	6/38	16
UNPC	18-90	64	16/64	25

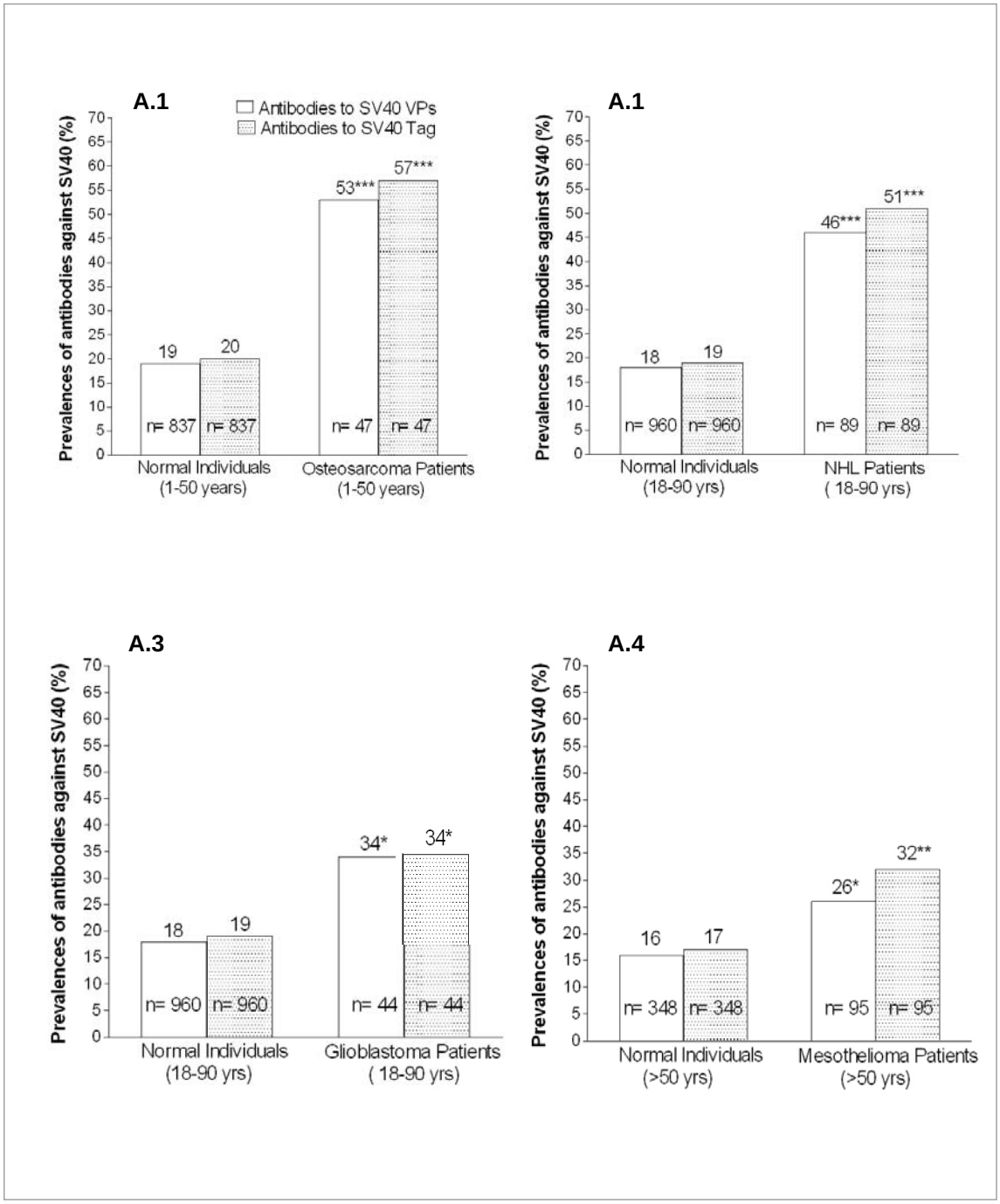
Table 4.10 Positive samples for SV40 VPs epitopes from breast cancers and UNPC patients.

Patients	Age	Number samples	Tag sieropositive	Tag sieropositive (%)
BC	18-90	38	8/38	21
UNPC	18-90	64	15/64	23

Table 4.11 Positive samples for SV40 Tag epitopes from breast cancers and UNPC patients.

Results obtained from the ELISA analyses indicate that sera from oncologic patients affected by osteosarcoma, glioblastoma, non-Hodgkin lymphoma and mesothelioma had a higher prevalence of antibodies reacting with SV40-VP and -Tag epitopes, than the seroprevalence detected in normal individuals (Figure 4.4). Specifically, SV40-positive sera from oncologic patients, belonging to 4 different cohorts had a higher prevalence compared to that of normal individuals. The control represented the same number of subjects, age and gender. In addition, statistically significant differences were confirmed by analyzing the prevalence of SV40-positive samples with a different number of subjects, where the number of normal individuals, employed as controls, was higher than that of the oncologic patients (Figure 4.4). Indeed, in the serum samples from osteosarcoma affected patients the prevalence was 53% and 57% for SV40 VP and Tag, respectively; in non-Hodgkin lymphoma, it was 46% for VP and 51% for the Tag; in glioblastoma, it was 34% both for VP and Tag, while the result was 26% for mesothelioma and 32% for VP and Tag, respectively (Figure 4.4A). Patients affected by mesothelioma were workers who had been exposed to asbestos, which is a natural oncogenic mineral with immunosuppressor activity. In our investigation, the prevalence of SV40 positive serum samples from mesothelioma patients was compared with the prevalence detected in healthy individuals, both those who had not been exposed and those who had been exposed to occupational asbestos fibres (Table 4.5). The two comparative analyses indicate that the prevalence of SV40 positive sera is higher in mesothelioma patients, and that these differences are statistically significant (Figure 4.4). The reduced prevalence of SV40-positive sera in workers previously exposed to asbestos, 9% for VP and 10% for Tag, who were very likely to have become immunodepressed by this mineral, was analyzed in comparison with the prevalence of SV40 positive sera detected in pregnant women (13%) (Figure 4.4C1/C2). Indeed, it is well established that pregnancy is a specific physiological state which renders women temporally partially immunodepressed.[168] Although prevalence in these two clusters is lower, compared to prevalence detected in normal individuals, the differences are not statistically significant (Figure 4.4C1/C2). As additional negative controls, sera from oncologic patients affected by breast cancers (BC) and undifferentiated nasopharyngeal carcinomas (UNPC) were analyzed by our ELISA assays, both for Tag and VP epitopes. Indeed, BC and UNPC are human neoplasms which are not associated with SV40, while

UNPC is linked to the herpesvirus EBV infection (Table 4.5). Serum samples from BC and UNPC affected patients reacted to SV40 epitopes with a prevalence (BC, 16% VP and 21% Tag; UNPC, 25% VP and 23% Tag) which did not differ substantially from those of healthy donors. Indeed, no statistically significant differences, determined by chi square testing, were observed between BC or UNPC and normal individuals (Figure 4.4B1/B2).



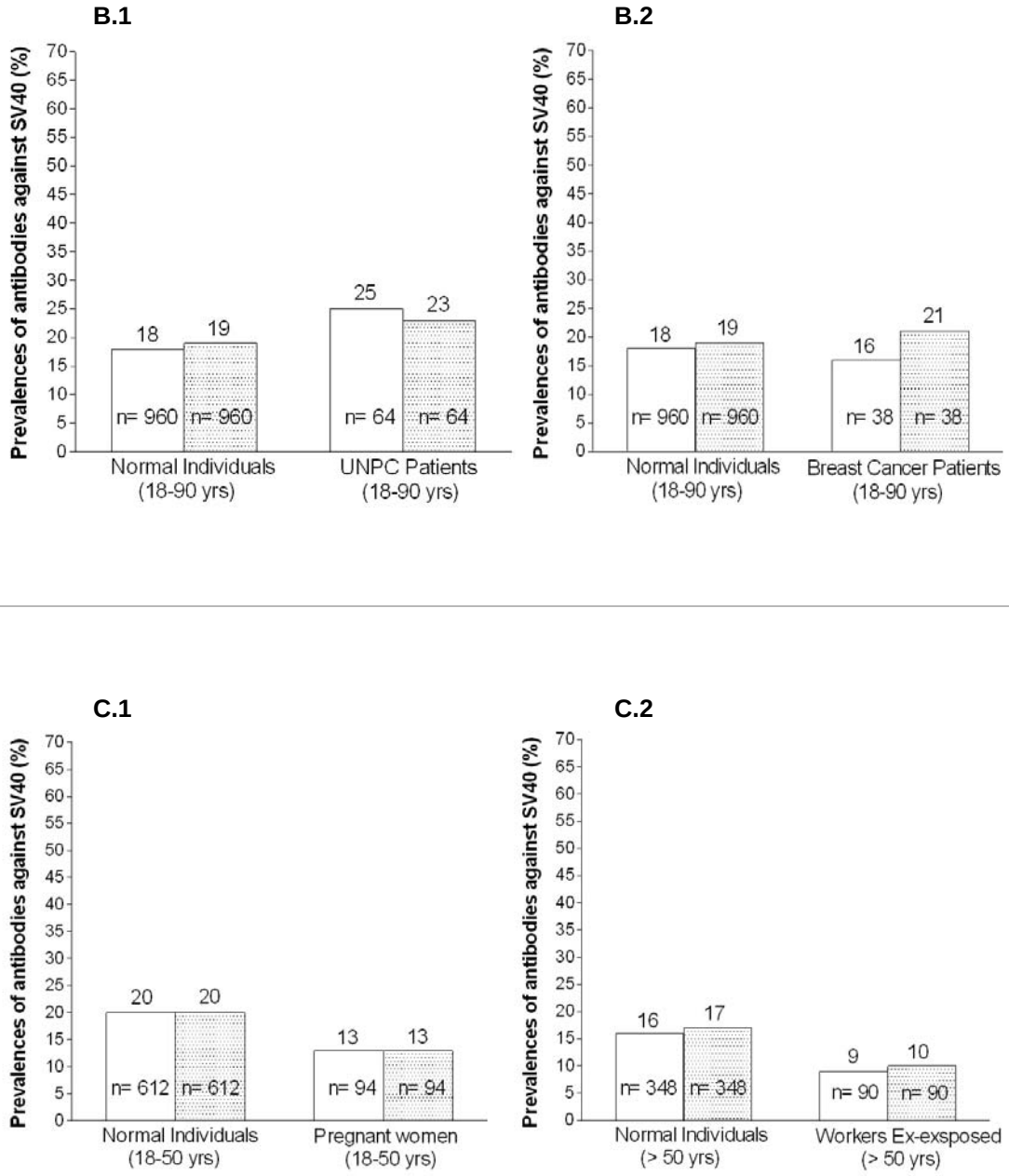


Figure 4.4 Prevalence of SV40 antibodies in serum samples from oncologic patients affected by osteosarcoma, NHL, glioblastoma and mesothelioma (panels A 1-4), UNPC and breast cancer (panels B 1-2) pregnant women and workers ex-exposed to asbestos fibres (panels C 1-2), in different age cohorts. Prevalence is expressed as a percentage, %. The number (n) of subjects in each group of age is indicated inside the bar. Percentage of serum antibodies against SV40 Tag and VP are marked with dotted and white bars, respectively. *p<0.05; **p<0.01; *** p<0.001.

In order to verify the antibody title, 24 SV40-positive sera, 8 from normal individuals and 16 from oncologic patients, i.e. 4 affected by malignant mesothelioma, 4 osteosarcoma, 4 non-Hodgkin lymphoma and 4 glioblastoma multiforme, were serially diluted from 1/20 to 1/640 and further investigated by indirect ELISA assays (Figure 4.5). Specifically, this ELISA assay indicated that the sera from normal individuals and oncologic patients affected by glioblastoma and mesothelioma carrying antibodies against SV40 remain positive at a 1/160 dilution, whereas those sera from patients with osteosarcoma and NHL are positive at lower dilutions, i.e. at 1/80. This result indicates that the title of SV40 antibodies in positive sera from normal individuals does not greatly differ from that detected in oncologic patients (Figure 4.5).

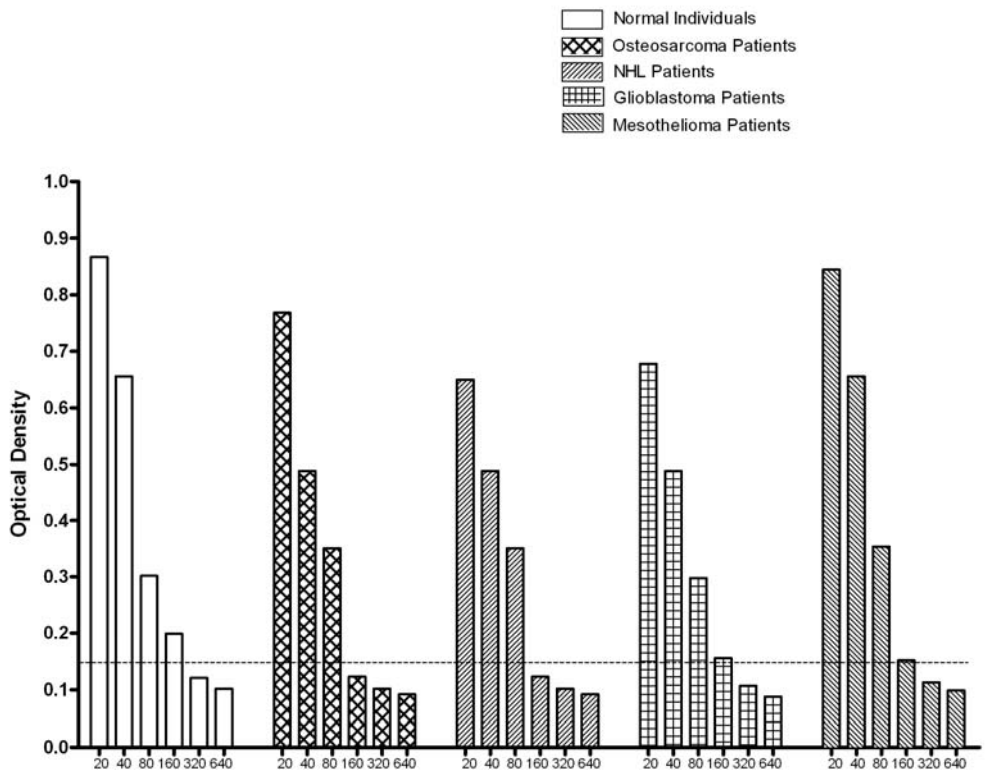


Figure 4.5 SV40 titer was determined, by the end point method, in 24serum samples with high O.D. Samples were serially diluted from 1/20 to 1/640 and then analyzed by the ELISA assay. In a typical ELISA test the general cut off, to consider SV40-positive a serum sample, was in the range of 0.120÷0.150, whereas samples with O.D. less than 0.120 are SV40-negative.

4.5 An antibody with neutralization activity

Since immune sera, containing antibodies against SV40, carry out neutralization activity against SV40 infectivity, an inhibition test was performed. To this purpose, among the 228 SV40 seropositive samples from normal subjects, six immune sera with a high O.D. were selected to test their ability to inhibit CPE in permissive CV1 SV40-infected cells. These sera were BKV and JCV negative, as determined by HAI test. The neutralization effect of immune sera is apparent in infected cells when the SV40 CPE is abolished or hampered (Tables 4.10 and 4.11).

Samples	OD(B)	OD(C)	Ab to SV40	Ab to BKV	Ab to JCV
C	0,310	0,387	+	-	-
D	0,403	0,398	+	-	-
E	0,427	0,421	+	-	-
F	0,599	0,655	+	-	-
G	0,617	0,518	+	-	-
H	0,722	0,723	+	-	-

Table 4.10 Positive samples for SV40 VPs and negative for BKV and JCV with relative value of optical density

Samples	OD(A)	OD(D)	Ab to SV40	Ab to BKV	Ab to JCV
C	0,321	0,309	+	-	-
D	0,401	0,405	+	-	-
E	0,413	0,420	+	-	-
F	0,634	0,643	+	-	-
G	0,764	0,754	+	-	-
H	0,876	0,876	+	-	-

Table 4.11 Positive samples for SV40 Tag and negative for BKV and JCV with relative value of optical density

The experiment was carried out together with SV40 seronegative and SV40 hyperimmune sera, used as negative and positive control sera, respectively. The CPE inhibition test was performed by mixing the serum sample with SV40 virions. Then, the mixture was employed as a viral inoculum to infect CV-1 permissive monkey kidney cells. In infection experiments, only titled SV40 viral stock was used as positive control, while a hyperimmune serum against SV40 with SV40 virions was employed as negative control (Table 4.12).

	Samples	Outcome
A	Negative control (CV-1 cells)	Monolayer intact
B	Positive control (CV-1 cells + SV40)	Monolayer completely disintegrated
C	CV-1 cells + SV40 + serum 1	Monolayer partially disintegrated (partially inhibited SV40 CPE)
D	CV-1 cells + SV40 + serum 2	Monolayer partially disintegrated (partially inhibited SV40 CPE)
E	CV-1 cells + SV40 + serum 3	Monolayer partially disintegrated (partially inhibited SV40 CPE)
F	CV-1 cells + SV40 + serum 4	Monolayer partially disintegrated (partially inhibited SV40 CPE)
G	CV-1 cells + SV40 + serum 5	Monolayer partially disintegrated (partially inhibited SV40 CPE)
H	CV-1 cells + SV40 + serum 6	Monolayer completely disintegrated (completely inhibited SV40 CPE)

Table 4.12 Neutralization of infections

Inhibition of SV40 CPE in infected cells indicated that the serum tested was able to neutralize SV40 infectivity. Indeed, among the six SV40-positive samples, one serum

(Figure 4.6/panel H) completely inhibited SV40 CPE, while five sera (Figure4.6/panels from C to G) only partially inhibited SV40 CPE. There was a correlation between the OD reading and the CPE inhibition activity of the immune sera. This assay detects functional antibodies to viral structural proteins, such as VP1, that are capable of neutralizing viral infectivity *in vitro* (Figure 4.6).

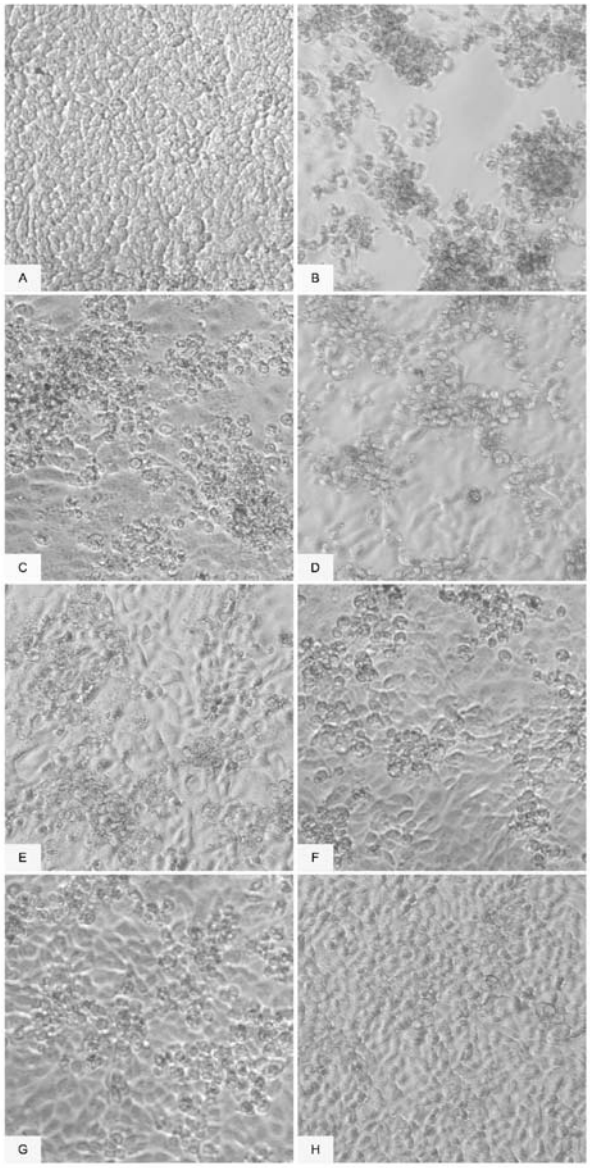


Figure 4.6 Panel A: Negative control represented by uninfected CV-1 cells. Panel B: Positive control by SV40 in CV-1 infected cells. Panels C-H. Distinct inhibition of CPE in SV40 infected CV-1 monolayers by human immune serum samples. Different IgG antibody title correlates with the CPE inhibition

Serum samples with high neutralizing activity correlate with the high OD determined in the anti-SV40 Tag and VPs ELISA tests (Figures 4.7). This result suggests that exposure to SV40 occurred in those immunized individuals and that the immune serum was elicited by SV40 infection and not by BKV or JCV.

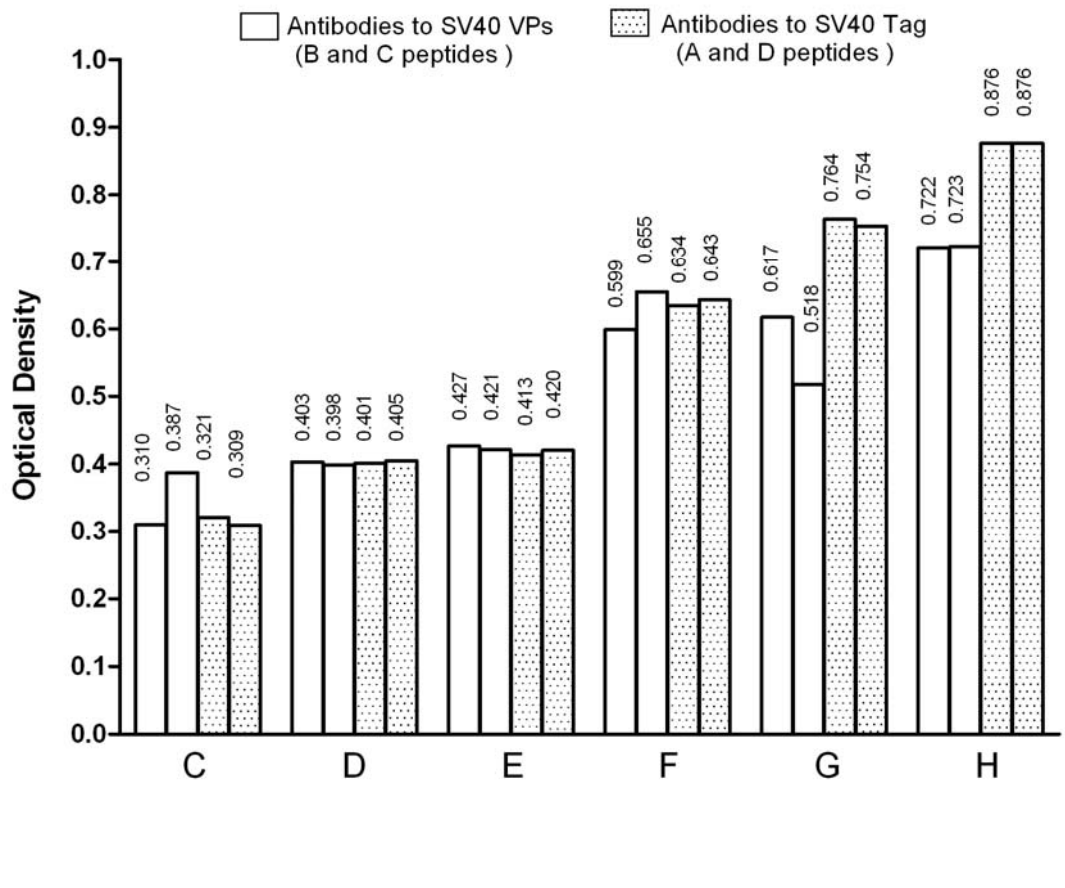


Figure 4.7 High O.D. values of the six serum samples, diluted 1/20, which inhibited at different extension the CPE of SV40-infected cells shown in figure 4.6.

Chapter 5

Discussion

5.1 Discussion

In our investigation, 1,276 serum samples from normal individuals were analyzed for their reactivity to SV40 epitopes of viral capsid proteins and Tag oncoprotein using indirect ELISA tests. The data indicate that the prevalence of SV40 antibodies in humans is in the range of 18% in healthy individuals from Italy. Taken together, data from our ELISA assays indicate that in normal individuals natural SV40 infection occurs with a lower prevalence, compared with the high infection prevalence spread over the two well characterized human BKV and JCV polyomaviruses, which is in the range of 60%-90%.[169] The period in which the first SV40 infection seems to occur is very early in childhood. IgM class SV40 antibodies, were detected in a 6-month-old child, suggesting that the first seroconversion begins in the perinatal age. The prevalence of anti-SV40 serum antibodies increases with age, reaching 22% (VPs) and

25% (Tags) in the cohort of children aged 1-10 years. Since SV40 seroconversion seems to occur at a very early age, one may postulate that this virus is transmitted through contact in the familial environment. Indeed, it has been shown that SV40 is present in the urine, stool, tonsil and blood specimens of children and adults suggesting that different ways of transmission, such as urine, oro-foecal, respiratory and haematic routes are responsible for SV40 infection.[170] Our data seem to confirm the results obtained by earlier studies on subjects administered with SV40-contaminated vaccines by different routes. Indeed, in these individuals, SV40 was detected/isolated after a number of weeks or days, either in their stools or from their tonsils depending on oral or nasal spray administration of contaminated vaccines.[171] The prevalence of IgG class anti-VPs and -Tag antibodies ranged from 19% to 20%, respectively in the group of 1-to-17-year-old subjects. In this cohort, there is a clear trend of increase in prevalence over the years, until the age of 10, with a peak of 22%-25% (Figures 4.2A and 4.2B), with an increase of SV40 antibody level determined by OD readings (Figure 4.3). The general trend in prevalence increases with age until fifty and then it declines in the cohort of individuals aged 51-65 years, while it increases again in the cohort of people aged 66-90 year-old. It is possible that in elderly individuals SV40 may reactivate or infect the host more efficiently. There are no significant differences among males, females and geographic regions for serum donors. The ratio between anti-Tag and anti-VPs antibodies was near to 1 in all the cohort of analyzed sera. Data on the inhibition of SV40 infectivity obtained with immune sera from normal subjects are of interest. There is a correlation between the grade of the inhibitory effect of SV40 infection by immune sera and their level of antibodies as determined by OD readings (Figures 4.5 and 4.6). The higher concentration of serum antibodies gave a stronger inhibition effect on SV40 infection in tissue culture. Since the selected SV40 immune sera were BKV and JCV negative, there is no possible cross-inhibition effect due to the presence of antibodies against these two human polyomaviruses. To further examine the serology of SV40-positive samples, endpoint titers were determined using an indirect ELISA assay. The highest endpoint titer was observed at a 1/160 dilution in 7 out of 8 (87%) sera from normal individuals, while only 6/16 (37%) sera from oncologic patients reached the highest endpoint titer at 1/160. Among the SV40-positive serum samples from oncologic patients, sera from osteosarcoma and non-Hodgkin lymphoma affected

patients carried the lowest OD reading compared to others from oncologic patients and normal individuals (Figure 4.5). The onset/progression of brain and bone tumors, mesothelioma and NHL, like other cancers, is associated with specific gene mutations. However, the agents responsible for the occurrence of mutations/chromosome alterations are poorly understood. SV40 was found to be associated with these specific human tumors. Indeed, SV40 sequences were detected at high prevalence in these neoplasms, indicating that this small DNA tumor virus is involved in the onset/progression of bone tumors.[172-177] Human osteosarcoma is the most common primary bone tumor affecting children and young adults. It comprises 56% of all bone cancers in individuals under the age of 20. Our immunologic data indicate that sera from patients affected by osteosarcoma have a high prevalence of antibodies against SV40 VP and Tag epitopes, with an average of 53% and 57%, respectively. This result confirms and extends the previous data on SV40 association with human osteosarcoma. Since the majority of these patients are teenagers or young adults, we may speculate that SV40 participates as a cofactor in the early phase of the cancinogenesis. Malignant pleural mesothelioma (MM) is a fatal human tumor MM onset is related to asbestos fibre inhalation, which is a tumorigenic natural mineral and an immunosuppressor agent.[178,179] Projections indicate that the number of patients who will die of MM in Western Europe, each year, will almost double over the next 20 years with a total of about 250,000 deaths until 2029 and then will decline.[180] Many reports indicate SV40, together with asbestos, as a co-carginogenic viral agent in the onset/progression of human MM.[181,182] It should be pointed out that other groups did not confirm the presence of SV40 footprints in human MM.[183] These conflicting data were interpreted by some authors as a result of PCR contamination with common laboratory plasmids containing SV40 sequences. While it is possible that some data may be due to laboratory contamination, it seems unlikely that all investigators who reported SV40-positive results contaminated their samples. The immunologic results presented herein confirm that this oncogenic virus is associated with human MM. In our investigation, sera from MM patients reacted with SV40 VP and Tag epitopes with a prevalence of 26% and 32%, respectively, which is a statistically significant difference compared to the prevalence detected in controls represented by sera from normal individuals or sera from workers who had previously been exposed to asbestos, and were not affected by

MM. NHL is a heterogeneous group of haematological malignancies which differ both biologically and clinically.[182] During the past 30 years, the reported incidence and death rate of the disease have increased strikingly worldwide. The reasons for the increase in incidences are not fully understood. However, many different viruses with oncogenic potential have been found to be associated with non-Hodgkin lymphoma, including SV40.[184] Our ELISA assays indicate that sera from patients affected by NHL contain antibodies reacting with SV40 epitopes from the late and early regions. Indeed, the prevalence of 46% and 51% of antibodies against VP and Tag epitopes were detected in these serum samples, suggesting that SV40 is associated with NHL. Brain tumors are rare neoplasms which represent 1% of human malignancies. These human tumors were among the first malignancies found to be associated with SV40 infection. Subsequently, after the advent of PCR technologies, many other studies reported the link. In our study, serum samples from patients affected by glioblastoma multiforme reacted in ELISA assays with specific SV40 antigens, from VP and Tag. Chi square testing has shown that the difference in the prevalence of SV40 antibodies, 34% both for VP and Tag, in these sera is statistically significant compared to that detected in sera from normal individuals. Data from our ELISA assay with sera from oncologic patients indicate that SV40 is associated with specific human malignancies, i.e. brain and bone tumors, MM and NHL, but not with other tumors used as negative controls such as UNPC and BC. The high prevalence of specific serum antibodies in the four malignancies studied herein indicates an association with SV40. However, it should be pointed out that the presence of SV40 antibodies at high prevalence in sera from oncologic patients is not proof of cause/effect in inducing specific human tumors by SV40. In our study, indirect ELISA assays, employing SV40 peptides from VP and Tag antigens, were set up for the detection of SV40 antibodies in human sera. After standardization, our ELISA tests gave reliable results, which can be obtained for many samples in a short period of time with affordable costs. This ELISA test can be shared with the scientific community and it may represent a standardized assay to study SV40 infection and its association with specific human tumors. Interestingly, our indirect ELISA test has been transferred to the analysis laboratory of our University Hospital, where the assay has been set up with an automatic processing system, obtaining the same results as the manual procedure. If the association of these malignancies with

SV40 is confirmed, it will be possible to set up new therapeutic approaches, such as the use of antiviral drugs against this small DNA tumor virus. In addition, new vaccines against SV40 could be prepared, which should be administered very early in life, in order to avoid SV40 infection occurring in the perinatal period. Indeed, one may speculate that early SV40 infection, when a child does not yet have a mature immune system, or because of impairment in the immune system of the adult, may allow this oncogenic virus to exert its tumorigenic potential, thus participating as a co-factor in the onset/progression of specific human cancers.

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